Studies on the use of modified live and killed porcine reproductive and respiratory syndrome virus vaccines to control virus shedding

by

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TABLE OF CONTENTS

ABSTRACT iv

CHAPTER 1. GENERAL INTRODUCTION 1

Introduction 1

Thesis Organization 3

CHAPTER 2. LITERATURE REVIEW 4

CHAPTER 3. A COMPARISON OF THE SHEDDING PATTERNS OF PIGS INFECTED WITH WILD-TYPE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) AND PIGS VACCINATED WITH A MODIFIED LIVE PRRSV VACCINE 32

Abstract 32

Introduction 32

Materials and Methods 34

Results 37

Discussion 44

References 48

CHAPTER 4. THE EFFECT OF KILLED VACCINE TREATMENT ON VIRUS SHEDDING IN PIGS PREVIOUSLY INFECTED WITH VIRULENT PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS 51

Abstract 51

Introduction 52

Materials and Methods 54

Results 58

Discussion 74

References 79

CHAPTER 5. GENERAL CONCLUSIONS 83

General Conclusions 83

Future Research 87

GENERAL REFERENCES 91

ACKNOWLEDGEMENTS 108

ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes persistent infection in swine. Commercial vaccines have been ineffective in decreasing the economic impact of this disease. Two studies were designed to determine if modified live and killed PRRSV vaccines could be used to control virus shedding.

The first study compared the virus-shedding patterns of wild-type and vaccine viruses. Two groups of pigs were infected intramuscularly with either wild-type (PRRSV VR-2332) or vaccine virus (PRIME PAC™, Schering-Plough). Oropharyngeal scrapings from both groups of pigs were collected weekly and assayed for virus and virus RNA beginning on day 49 post infection (p.i.). Sentinel pigs were placed in contact with both groups on 77 and 91 days p.i. There was a difference in the virus shedding patterns of wild-type and vaccine viruses after 70 days p.i. Virus RNA was not detected in oropharyngeal scrapings of any pig infected with vaccine virus after 70 days p.i., but it was detected in 2 pigs infected with wild-type virus between days 70 and 105 p.i. Virus RNA was detected in tonsils at necropsy on day 109 in 3 pigs infected with vaccine virus and in 2 pigs infected with wild-type virus. Virus was not isolated from any oropharyngeal sample. Sentinel pigs did not seroconvert as indicated by the enzyme-linked immunosorbent assay. The presence of virus RNA in tonsils of both groups at necropsy suggests that virus remained in the pigs but did not transmit to the sentinels. Whether virus shedding can be reinitiated needs to be further investigated.

In the second study, two separate experiments were conducted to determine if virus shedding in pigs previously infected with virulent PRRSV could be reduced by enhancing immune response through the use of a killed vaccine (KV) (PRRomiSe®, Intervet Inc.). In the first experiment, infected pigs were vaccinated with KV on days 14 and 28 p.i. The viremias and serum virus neutralizing (SN) antibody responses of KV-treated infected pigs and infected control pigs were compared. The second experiment was conducted in an identical manner to the first experiment with the following exceptions. Two vaccine treatment groups were used. One group of infected pigs was vaccinated with KV on days 7 and 21 p.i., whereas the second group was vaccinated on days 14 and 28 p.i. Oropharyngeal scrapings were collected on days 42 and 56 p.i. and tested for virus and virus RNA. The number of PRRSV specific interferon gamma (IFN-γ) producing cells was monitored biweekly, starting on day 42 p.i. There was no difference in the magnitude and duration of viremia between the KV-

treated infected group and the non-treated infected control group. The mean SN antibody titer of the KV-treated infected group was significantly greater ($p \leq 0.05$) than that of the infected group on day 42 p.i. in the first experiment, and on days 42 and 56 p.i. in the second experiment. In the second experiment, the mean number of IFN-γ producing cells was slightly higher in KV-treated infected groups than that of the non-treated infected group on days 42 and 63 p.i. These observations suggest that KV treatment did not reduce virus shedding even though infected pigs responded immunologically to the KV treatment.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense, single-stranded RNA virus in the order *Nidovirales* and the family *Arteriviridae*. Other viruses in this family are equine arterivirus, lactate dehydrogenase elevating virus and simian hemorrhagic fever virus (Cavanagh, 1997). Clinical signs caused by PRRSV infection are reproductive failure in dams, respiratory disease in grower and finisher pigs, and increased pre-weaning mortality (Loula, 1991). An important characteristic of this virus is its persistent nature (Wills et al., 1997c). This characteristic has interfered with current control and elimination programs.

The disease was first reported in the late 1980s and referred to as the mystery swine disease (Hill, 1990). Initially, the etiology was unknown and various agents were considered to be the cause of this disease. The causative agent remained unidentified until 1991, when researchers in the Netherlands isolated an agent from infected pigs by using porcine alveolar macrophages. The agent was characterized as a virus, and later named the Lelystad virus (LV) (Wensvoort et al., 1991). The clinical signs caused by the LV were reproduced experimentally when pre-weaning pigs and pregnant sows challenged with the virus developed a syndrome resembling those of the mystery swine disease (Terpstra et al., 1991;Wensvoort et al., 1991). In the U.S.A., the first isolation of PRRSV was made in 1992 using the continuous cell line, CL2621 (Collins et al., 1992).

Since its first emergence, PRRSV has continued to cause disease that has a significant economic impact on the swine industry worldwide. Polson et al. (1992) estimated that losses due to decreased liveborn litter size, increased preweaning mortality and a decreased farrowing rate have reduced profits by $236 per sow in inventory. Losses due to an increase in days to market, from 14 to 30 days, have resulted in increased costs from $7.50 to $15.00 per marketed pig (Dee and Joo, 1993). Veterinarians and producers have become increasingly convinced that it is economically advantageous to maintain herds free of PRRSV. Therefore, several management protocols have been developed to eliminate PRRSV from herds. They include total depopulation followed by repopulation with PRRSV-free replacement pigs (Andreasen et al., 1998), partial herd depopulation such as the depopulation of nursery units (Hassing et al., 2000), Isowean (Christianson et al.,

1994; Rajic et al., 1999), test and removal (Dee et al., 2001), temporary herd closure (Henry et al., 2000; Torremorell et al., 2000), and total herd vaccination (Philips et al., 2000).

Vaccines for PRRSV are currently available, including a killed vaccine (KV) from Intervet, Inc. (PRRomiSe®) (Christensen et al., 1998) and a modified live virus vaccine (MLV) from Boehringer Ingelheim Vetmedica, Inc. (RespPRRS/repro®) (Gorcyca et al., 1997). However, there is controversy concerning the effectiveness of both vaccines. For example, KV induces a poor immune response even after two immunizations. Modified live PRRSV vaccine is unable to stimulate a strong cellular immune response or to induce significant titers of serum virus-neutralizing antibodies. Moreover, PRRSV MLV vaccinated pigs are more likely to spread the vaccine virus to non-infected pigs.

It is perceived that the use of PRRSV MLV may have resulted in the infection of many herds. By using a site-restriction marker specific for vaccine virus, Mengeling et al. (1999c) demonstrated that vaccine virus was spread after the introduction of PRRSV MLV (RespPRRS/repro®) in 1994. None of 25 field PRRSV isolates collected prior to the introduction of MLV was a vaccine virus. In contrast, 21 of 25 field PRRSV isolates collected after the introduction of MLV were vaccine viruses. Torrison et al. (1996) found that pigs vaccinated with PRRSV MLV shed vaccine virus to susceptible pigs. The susceptible pigs became infected after exposure. In Denmark, acute PRRS-like syndromes characterized by an increase in numbers of abortions and stillborn piglets and increased mortality in nursing pigs were reported in previously PRRSV seronegative herds following the introduction of PRRSV MLV into the country. It was verified that vaccine virus spread among these herds and caused the disease (Botner et al., 1997).

It is possible that PRRSV vaccines could be used more effectively. For example, MLV could be used in replacement pigs prior to their introduction into a herd. The introduction of MLV in acclimatization might be useful as MLV might not be as persistent as wild-type virus infection. Srinivasappa et al. (1999) evaluated vaccine virus transmission over a 60-day period and found that vaccine virus was transmitted to sentinel pigs though day 39 post infection (p.i.), but not thereafter. In contrast, a study by Wills et al. (2000) suggested that wild-type virus is commonly transmitted through 60-70 days p.i. Taken altogether, the vaccine virus appears to be shed for a shorter period of time than wild-type virus. However, there has not been a simultaneous comparison of shedding patterns between wild-type and vaccine viruses.

Killed vaccine is not efficacious. Osorio et al. (1998) indicated that PRRSV negative pigs vaccinated twice with KV might not have resulted in the induction of an antibody response. However, KV enhanced SN antibody response when it was used in pigs previously infected with PRRSV (Trayer, 1999). The enhancement of SN antibody response was also demonstrated when KV was administered to pigs previously vaccinated with MLV (Baker et al., 1999).

The studies described in this thesis were designed to compare the shedding patterns of wild-type and vaccine viruses and to determine if virus shedding in pigs previously infected with virulent PRRSV could be reduced significantly or inhibited by enhancing immune response through the use of a commercial KV. The results from this research could contribute to the control and elimination of PRRSV.

Thesis Organization

This thesis is organized into five chapters, and begins with an abstract that provides a summary of the general objectives, results and conclusions. The format of the thesis follows that of Veterinary Microbiology. The first chapter contains the general introduction and thesis organization. The second chapter contains the literature review. The third chapter is a paper that will be submitted for publication in the Veterinary Record, and is entitled "A comparison of shedding pattern of pigs vaccinated with porcine reproductive and respiratory syndrome virus modified-live virus vaccine and pigs infected with wild-type virus." The fourth chapter is a paper that will be submitted for publication in Veterinary Microbiology, and is entitled "The effect of killed vaccine treatment on virus shedding in pigs previously infected with virulent porcine reproductive and respiratory syndrome virus (PRRSV)." This researcher is the primary author of both manuscripts. The co-authors are D. L. Harris, K. B. Platt, P. G. Halbur and M. Torremorell. The fifth chapter includes a general conclusion summarizing the results, and recommendations for future research. References for the first, second and fifth chapters are included in the general references.

CHAPTER 2. LITERATURE REVIEW

Porcine reproductive and respiratory syndrome (PRRS)

In 1987, a swine disease of unknown etiology causing reproductive and respiratory disorders was reported for the first time in the swine-producing areas of North Carolina, Minnesota and Iowa of the U.S. (Hill, 1990; Keffaber, 1989; Loula, 1991). These disorders were referred to as the mystery swine disease since no definitive cause could be assigned to them. The characteristics of the disease were: a) late-term abortions; b) an increase in the number of stillbirths, mummified fetuses and weak-born pigs; d) irregular return to service; e) high death rate of pigs prior to weaning; and f) pneumonia in young pigs (Collins et al., 1992; Hill, 1990; Keffaber, 1989; Loula, 1991; Wensvoort et al., 1991). The mystery swine disease was reported as epidemics in Canada by the end of 1987 (Dea et al., 1992).

A syndrome similar to the mystery swine disease was first reported in June 1990 in Germany (Meredith, 1995). The disease affected more than 80% of herds. The disease appeared in the Netherlands from the winter of 1990 through the spring of 1991. It was perceived that the disease spread from Germany (Meredith, 1995). The clinical syndrome of this disease was reported for the first time in Spain in 1991 (Plana et al., 1992), where it occurred in herds that received piglets from Germany. In England, the disease was first recognized in Humberside during 1991, where it was initially called the blue ear disease (Meredith, 1995). The disease spread further to Scotland in 1991 due to the movement of carrier pigs and contaminated semen obtained from the U.K. A disease clinically resembling these reports was also reported in 1991 in the region of Brittany, France (Baron et al., 1992). The first case in Denmark appeared in 1992 (Botner et al., 1994), where it was most likely spread via airborne transmission from Germany.

Etiology

The causative agent of PRRS was unknown when the disease first emerged. Several infectious organisms were considered as causative agents, including encephalomyocarditis virus, porcine parvovirus, pseudorabies virus, swine influenza virus, an influenza-like virus, bovine viral diarrhoea, hog cholera virus, porcine enterovirus and chlamydiae (Wensvoort, 1993). However, none of these agents were proven to cause PRRS.

The causative agent remained unidentified until 1991, when researchers in Lelystad, The Netherlands, isolated an agent from infected pigs by using porcine alveolar macrophages. The agent was identified as a virus, which was later called the Lelystad virus (LV) (Wensvoort et al., 1991). The clinical signs caused by the LV were reproduced experimentally when pre-weaning pigs and pregnant sows challenged with the virus developed a syndrome resembling that of the mystery swine disease (Terpstra et al., 1991; Wensvoort et al., 1991). Soon after its discovery in the Netherlands, this virus was isolated in several countries throughout Europe (Baron et al., 1992; Plana et al., 1992). Specific antibodies against the LV were also detected in pigs with mystery diseases from various countries. The clinical signs caused by the LV were reproduced repeatedly in experimental pigs by several investigators (Plana et al., 1992; Terpstra et al., 1991). Therefore, the LV was verified to be the causative agent of the mystery swine disease. In Europe, the name "porcine epidemic abortion and respiratory syndrome" (PEARS) virus was proposed as the name for this mystery virus (Terpstra et al., 1991).

In the U.S.A, an attempt to isolate the causative agent of the mystery disease was successful in 1992 (Collins et al., 1992). Infected piglets were retrieved from farms experiencing clinical signs of the disease and the affected tissues, such as lungs, tonsils and lymph nodes, were collected and homogenated. Gnotobiotic pigs were then exposed to the tissue homogenates and killed at 8 days post infection (p.i.). Their lungs, tonsils and lymph nodes were collected, homogenized and filtered through 0.22 μm. The filtrates were inoculated onto a monolayer of CL2621 cells. After the second blind passage, cytopathogenic virus was isolated. Koch's postulates were fulfilled when the gnotobiotic pigs exposed intranasally to the isolated virus developed clinical signs similar to those of pigs exposed intranasally to the homogenized tissues. The virus was reisolated from both groups of pigs, but not from sham inoculated controls (Collins et al., 1992). The virus was submitted to the American Type Culture Collection as VR-2332 (ATCC VR-2332), and named "swine infertility and respiratory syndrome" (SIRS) virus.

After the causative agent was identified for this virus, the name "porcine reproductive and respiratory syndrome virus" (PRRSV) was adopted at the First International Symposium on SIRS/PRRS in Minnesota, U.S.A. (Goyal, 1993). At this meeting, the name porcine reproductive and respiratory syndrome (PRRS) was

adopted internationally as the clinical syndrome characterized by reproductive disorders in female pigs and respiratory distress, including pneumonia in younger pigs.

Porcine reproductive and respiratory syndrome virus was assigned to the genus *Arterivirus*, family *Arteriviridae* of the newly created order, *Nidovirales* (Cavanagh, 1997; Meulenberg, 2000). Members of the *Arteriviridae* include lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus (SHFV). Initially, PRRSV, EAV, LDV and SHFV were placed in the family *Togaviridae* because of their similarity in size and structure. However, during the 10th International Congress of Virology in 1996, a new viral order, the *Nidovirales* was established in which the viruses of the families *Coronaviridae* and *Arteriviridae* were incorporated. The *Coronaviridae* were distributed into 2 genera, the *Coronavirus* and *Torovirus* (Cavanagh, 1997). Serological cross-reaction was not found between PRRSV and other arteriviruses and coronaviruses (Wensvoort et al., 1992b). Genomic studies have revealed that there are two distinct genotypes of PRRSV which represent the prototypes of the North American and the European isolates (Meng et al., 1995a; Murtaugh et al., 1995; Nelsen et al., 1999). Both isolates are similar in morphology, structure, and organization of the genome.

Morphology and physical properties

Porcine reproductive and respiratory syndrome virus is a small, enveloped virus containing a positive-sense and single-stranded RNA genome. The virus particle is approximately 45-55 nm in diameter (Benfield et al., 1992; Mardassi et al., 1994). It has a nucleocapsid core of approximately 25-35 nm in diameter that is surrounded by a lipid bilayer membrane (Benfield et al., 1992).

The buoyant density of PRRSV is approximately 1.18 g/ml in a cesium chloride gradient and 1.18-1.23 g/ml in a sucrose gradient (Benfield et al., 1992). The virus is heat labile and can be inactivated in 24 hours at 37°C, or in 45 minutes at 56 °C. The virus is stable for 1 month at 4°C and for 4 months at –70 °C. It is inactivated in media with a pH lower than 5 or greater than 7.

Porcine reproductive and respiratory syndrome virus does not survive well outside the host. The virus does not persist on fomites at ambient temperatures. It remains active when keep moist (Zimmerman et al., 1997b). Virus infectivity is destroyed at 37 C within 48 hours and at 56 C within 45 minutes. It retains its

infectivity at temperatures of 4°C for 1 month and –70°C for 4 months (Benfield et al., 1992). Virus is stable at pH 6.5-7.5, but exposure to a pH below 6 or over 7 will reduce its infectivity over 90% (Benfield et al., 1992).

Genomic organization

The complete genomes of the North American (VR-2332) and LV isolates of PRRSV have been sequenced (Allende et al., 1999; Wooton et al., 2001; Yuan et al., 2001). They are approximately 15 kilobases in length and have a similar genomic organization as other arteriviruses.

Eight open reading frames (ORFs) have been identified in the PRRSV genome (Conzelmann et al., 1993; Meulenberg, 2000). Open reading frame 1 encodes nonstructural proteins. The ORF 1 of PRRSV is approximately 80% of the entire genome. It is further divided into ORF 1a and 1b. Both ORFs are located at the 5′ end of the genome and function to encode the RNA-dependent RNA polymerase known as RNA replicase (Meulenberg, 2000). The other 6 ORFs (ORFs 2-7) encode six putative structural proteins with molecular masses of approximately 30, 45, 31, 25, 19 and 15 kD, respectively (Meulenberg et al., 2000) and are located at the 3′end of the genome. The proteins encoded by ORFs 2-5 are glycosylated and are located in the viral envelope. Glycoprotein 3 is the highest glycosylated protein compared to other glycoproteins (Gonin et al., 1998). The ORF5 is the most variable region. It encodes GP5 that is belived to be correlated with neutralizing activity. Since Mabs directed to the GPs 4 and 5 neutralized viruses *in vitro*, it is suggested that epitopes on GPs 4 and 5 may be targets of neutralizing antibodies. However, Gonin et al. (1999) reported that GP5 is more correlated with neutralizing activity than GP4. Open reading frame 6 is the most conserved region of *Arterivirus* genomes and encodes the matrix (M) protein, which is non-glycosylated. Yang et al. (2000) reported that epitope on M protein may be a target of neutralizing antibody also, since monoclonal protein (Mab) representing an epitope on the M protein has neutralizing activity. Open reading frame 7 encodes the nucleocapsid (N) protein. Since most Mabs were directed against the N protein, it is suggested that it is the most immunodominant protein.

Genetic variation

There are two distinct genotypes of the PRRSV, which represent the North American and the European isolates (Allende et al., 1999; Meng et al., 1995a; Murtaugh et al., 1995). Studies of the genomic sequence have revealed that PRRSV has evolved independently on separate continents from a common ancestor (Murtaugh et

al., 1995; Nelsen et al., 1999). Genetic comparisons between PRRSV and other members of the *Arteriviridae* suggest that PRRSV might be more closely related to LDV than EAV or SHFV (Meng et al., 1995a; Murtaugh et al., 1995; Nelsen et al., 1999).

Extensive amino acid sequence analysis of PRRSV isolates has revealed high genetic variation between the North American and European isolates, as well as among the North American isolates themselves (Allende et al., 1999; Andreyev et al., 1997; Meng et al., 1995a, 1995b, Morozov et al., 1995; Yang et al., 1998). The comparative sequence analysis of ORFs 2 to 7 indicates that there is some degree of amino acid identity between the VR-2332 and LV isolates (Meng et al., 1995a; Murtaugh et al., 1995). The amino acid identities between VR-2332 and LV isolates are 63%, 58% and 68% for proteins encoded by ORFs 2, 3 and 4, respectively. The amino acid identity of the protein encoded by ORF 5 is 51-59%. In contrast, the amino acid identity of the protein encoded by ORF 6 is 70-81%. The amino acid identity of protein encoded by ORF 7 is 59-65%. The major difference is observed primarily in ORF 1 (Allende et al., 1999), in which 47% of the amino acid identity occurs in a polyprotein encoded by ORF 1. Phylogenetic studies of ORFs 2-7 indicate that on average amino acid sequence of the GP5 of the LV isolate is 35% different from that of the North American (VR-2332) isolate (Kapur et al., 1996).

Among the North American isolates, a statistical comparison demonstrated that the genetic distances between isolates ranged from 7.9% to 20.5% (Kapur et al., 1996). The amino acid identity is 91-99%, 86-98%, 92-99%, and 88-97% for proteins encoded by ORFs 2, 3, 4, and 5, respectively. The amino acid identity is 95.4-100% for proteins encoded by ORFs 6 and 7.

Antigenic variation

The first evidence of antigenic difference among PRRSV isolates was provided by Wensvoort et al. (1992a). The immunoperoxidase monolayer assay (IPMA) was used to test the reactivity of polyclonal antisera collected from pigs exposed to the North American or European PRRSV isolate to wild-type PRRSV collected from the U.S. and Europe. The investigators found that the titer was high when testing the North American antisera with the U.S wild-type PRRSV. In contrast, the titer was low when testing the North American antisera with the European wild-type PRRSV.

Yang et al. (1999, 2000) categorized PRRSV by developing an virus epitope marker system based on reactivity to Mabs and the antigenic divergence of PRRSV isolates. These investigators generated a panel of Mabs directed against epitopes on N protein and were able to categorize 70 field PRRSV isolates into 5 antigenic groups (Yang et al., 1999). Subsequently, these investigators generated more Mabs that recognize epitopes on GP3, GP5 and M proteins and were able to further categorize antigenic groups I and II into 9 and 4 subgroups, respectively (Yang et al., 2000). It is notable that 3 Mabs representing epitopes on the GP5 and M proteins have neutralizing activities and viruses in each antigenic group vary in their susceptibility to neutralization.

Clinical signs

Clinical signs observed in PRRSV-infected pigs vary depending on a number of factors such as virus strain, age, sex, and reproductive status of the pig. Typical clinical signs usually seen in the acute phase of classical PRRSV infection include disorders of the respiratory and reproductive systems (Hill, 1990). Lack of appetite, lethargy, depression and pyrexia are generally noticeable. The prominent clinical sign in younger pigs is respiratory distress. An increase in pre-weaning mortality is often observed at nursery age. In breeding pigs, a lack of appetite along with respiratory signs such as sneezing, coughing and pneumonia are observed. The major effects in breeding pigs are reproductive loss, including late-term abortion, repeated estrous, premature farrowing, mummified fetuses, stillbirths and weakborn piglets.

Boars are also affected by PRRSV infection, often developing mild respiratory disease (Swenson et al., 1994). Libido is not affected by the PRRSV infection, however, the quality and volume of semen from PRRSV-infected boars are decreased (Christopher-Hennings et al., 1997). Abnormality in sperm morphology and reduction in motility are also observed in infected boars. Immature sperm cells infected with PRRSV increase significantly in semen of infected boars (Sur et al., 1997).

There are marked differences in virulence between U.S. and European PRRSV strains as well as among U.S. isolates. Two studies were conducted by Halbur et al. (1995b, 1996b) using 25 4-week-old and 146 5-week-old cesarean-derived, colostrum-deprived (CDCD) pigs. Pigs were inoculated with LV or 1 of 2 U.S. isolates of PRRSV (VR-2385 and VR-2431) in the first study (Halbur et al., 1995b), and 9 U.S. isolates in the second study (Halbur et al., 1996b). Marked differences in clinical signs as well as pathogenicity were observed

in both studies. The LV and VR-2431 strains caused mild transient pyrexia, including dyspnea and tachypnea. The VR-2385 strain induced more severe respiratory signs including labored respiration and lethargy. The mean gross lung lesion score estimating the percentage of lungs affected by pneumonia at 10 days after inoculation ranged from 6.8% for LV to 64.2% for VR-2385 (Halbur et al., 1995b). The mean gross lung lesion score among U.S. isolates ranged from 16.7% to 62.4% in the second study (Halbur et al., 1996b). The marked difference in pathogenicity would explain the marked difference in clinical signs.

A study by Mengeling et al. (1996) demonstrated differences in virulence among strains of PRRSV for their ability to cause reproductive failure in pregnant pigs. In the study, gilts were infected with 4 strains of PRRSV (vaccine strain, VR-2385, VR-2431 and NADC-8) at approximately 90 days of gestation. The number of dead-fetuses was used as a measure of virulence for different strains. All gilts were viremic after inoculation and all isolates crossed the placenta as evidenced by virus isolation from fetuses. The number of dead-fetuses ranged from 0 for 4 gilts in the control group and 4 gilts exposed to the vaccine strain, to 38 for 4 gilts exposed to PRRSV strain NADC-8.

Pathogenesis

The PRRSV enters the host through the mucosal surface of the respiratory tract (Meulenberg, 2000). Pulmonary alveolar macrophages (PAMs) are believed to be the primary site of replication. The virus can infect pulmonary intravascular macrophages (PIMs). The virus is transported from macrophages to regional lymph nodes as cell-free or cell-associated PRRSV for secondary replication. The second replication site can be tonsil, lung, spleen and lymph nodes. Infected pigs become viremic and shed the virus as quickly as 12-24 hours post-exposure. PRRSV can also be found in heart, thymus, spleen, brain, testes and ovaries (Duan et al., 1997; Halbur et al., 1996a, 1996b; Rossow et al., 1994a, 1994b, 1998, 1999; Sur et al., 1997, 2001; Thanawongnuwech et al., 1997). The virus is detected in nasal discharge, fecal samples and oropharyngeal scrapings as well (Rossow et al., 1994a; Wills et al., 1997b). The infection results in interstitial pneumonia, myocarditis, rhinitis, vasculitis and lymphadenopathy (Halbur et al., 1996a; Rossow, 1998).

Gross and microscopic lesions of PRRSV infection have been studied by several investigators in which various PRRSV isolates were used (Halbur et al., 1996a, 1996b; Rossow et al., 1994a, 1994b). Gross lung lesions vary from none to diffuse consolidation. The affected lobes are mottled and diffusely tan in color.

Interstitial pneumonia is the most common form of lung lesion observed in both naturally and experimentally infected pigs. Microscopic lung lesions are characterized by a thickening of the alveolar septa with infiltration of macrophages and mononuclear cells. Necrotic cells and dead cell debris are also observed in the alveoli. The lymph nodes of both naturally and experimentally infected pigs are markedly enlarged (Halbur et al., 1996a, 1996b). The microscopic lesions in the lymph nodes are characterized by germinal center hypertrophy and hyperplasia, and necrosis of the germinal center. Lymph node lesions in congenitally infected pigs are characterized by cell necrosis, and germinal center cell hypertrophy and hyperplasia (Rossow et al., 1994b). Follicular hypertrophy is observed in tonsils and Peyer's patches. Vasculitis is observed in vessels of all sizes and is characterized by the infiltration of macrophages, lymphocytes and mononuclear cells. Infiltration of lymphocytes and mononuclear cells is observed in the heart and brain lesions, and causes myocarditis and meningoencephalitis (Rossow et al., 1994a). The virus is also found in microglia and neurons of brains in infected pigs, especially young pigs (Rossow et al., 1999; Thanawongnuwech et al., 1997).

The PRRSV infection can induce apoptosis as characterized by chromatin condensation, intact cell and nucleosome ladder formation, and rRNA degradation (Sirinarumitr et al., 1998; Suarez et al., 1996; Sur et al., 1997, 1998). The PRRSV ORF5 product appeared to induce apoptosis in infected cells (Suarez et al., 1996). Apoptosis was detected predominantly in mononuclear cells, alveolar, and pulmonary intravascular macrophages of lungs, and macrophages in lymph nodes of infected pigs. The PRRSV-induced apoptosis was also demonstrated in testicular epithelial germ cells of infected boars (Sur et al., 1997). The majority of apoptotic cells were uninfected bystander cells (Sirinarumitr et al., 1998). The induction of apoptosis by PRRSV infection might be significant in PRRSV pathogenesis that could be an explanation for the dramatic reduction in the number of alveolar macrophages during infection.

Anti-PRRSV specific antibodies have been implicated in the pathogenesis of PRRS through a mechanism called a antibody-dependent enhancement (ADE) (Yoon et al., 1996, 1997). The replication of PRRSV is enhanced when PRRSV specific antibodies are present. Antibody-dependent enhancement is relevant in PRRSV pathogenesis because macrophages, which are the main target for PRRSV in pigs, can uptake the antibody-virus complexes via Fc receptors. Yoon et al. (1996) indicated that ADE of PRRSV infection has a potential to contribute to the severity of disease and to increase the susceptibility of pigs to PRRSV infection.

The antibodies causing ADE are speculated to be directed to GP 5 (Yoon et al., 1996). The heterogeneity of PRRSV may produce variation in the susceptibilities of PRRSV isolates to ADE (Yoon et al., 1997).

Persistent infection

Zimmerman et al. (1992) first demonstrated that PRRSV causes a persistent infection in experimentally infected pigs. An experimentally infected sow transmitted the virus to 3 susceptible pigs up to day 99 p.i. Subsequently, Albina et al. (1994) found that PRRSV-negative pigs became infected with the virus when commingled with pigs infected 154 days previously. Furthermore, Benfield et al. (1997) demonstrated that virus RNA was detected by PCR for up to 210 days post partum in pigs farrowed from gilts exposed to PRRSV during late gestation. A study by Horter et al. (2000) demonstrated that 100% of experimentally infected pigs carried PRRSV on 63 days p.i. The carrier rate dropped to 90% by 105 days p.i. The virus does not seem to persist for the life of pigs. Allende et al. (2000) suggested that the virus is eventually eliminated from the body of infected animals by 150 days p.i. or shortly thereafter.

The mechanism of persistent infection is not clearly understood. However, PRRSV is often isolated from tonsils during the chronic phase of infection. Therefore, the tonsil might be a possible site of persistent infection (Benfield et al., 1999).

Following virus exposure, PRRSV-infected pigs shed the virus for an extended period of time. Virus is detected in saliva, nasal secretions, urine, semen, feces and mammary secretions. Virus shedding occurs from many sites and is perhaps intermittent. For example, Wills et al. (1997b) detected virus in saliva up to 42 days p.i. and in urine up to 14 days p.i. In contrast, virus was detected in urine of a single pig among 9 pigs sampled on day 28 (Rossow et al., 1994a). Virus was recovered from nasal swabs in 3 of 5 pigs on day 21, but it was not recovered from any sample collected on 1, 4, 7, 14, and 28 days p.i (Wills et al., 1997b).

Wills et al. (1997b, 1997c) conducted studies to monitor the virus shedding pattern of pigs infected with PRRSV VR-2402 over a 124-day period in the first study and over a 210-day period in the second study. In both studies, virus shedding was monitored by collecting oropharyngeal scrapings beginning 55 days p.i. In the first study, PRRSV was recovered from oropharyngeal scrapings in 2 of 4 pigs between 55 and 84 days p.i. No virus was detected after day 84 (Wills et al., 1997b). In the second study, PRRSV was recovered from

oropharyngeal scrapings from 3 of 4 pigs between 56 and 84 days p.i. and from a forth pig up to 157 days p.i (Wills et al., 1997c).

Boars shed the virus in semen for variable periods of time following infection (Christopher-Hennings et al., 1995a, 1995b; Swenson et al., 1994; Yaeger et al., 1993). Swenson et al. (1994) found that 1 of 4 experimentally infected boars shed infectious virus in their semen for 43 days after infection. In another study, virus RNA was detected up to 92 days p.i. in semen of experimentally infected boars (Christopher-Hennings et al., 1995b). Transmission of the virus to females by artificial insemination with undiluted semen from experimentally infected boars has been demonstrated (Yaeger et al., 1993).

Wagstrom et al. (1998) found virus in milk and colostrum of gilts following vaccination with MLV or challenge with wild type virus in the third semester of gestation. Virus was isolated in 2 of 2 PRRSV challenged pigs, and in 1 of 2 MLV vaccinated pigs during the first 18 days of lactation.

The effect of stress on virus shedding by persistently infected pigs is unclear. Albina et al. (1994) reported that virus could be reactivated following stress induction. In the study, sows were infected experimentally at 90 days of gestation. Age-matched sentinel pigs were commingled with piglets weaned from infected sows. Virus transmission to susceptible pigs stopped on day 60 p.i. However, virus transmission to sentinel pigs took place again on day 154 following stress by forced movement and administration of corticosteroid. In contrast, Christopher-Hennings et al. (1995a) and Swenson et al. (1995b) were unable to recover virus following stress induction.

Virus transmission

Porcine reproductive and respiratory syndrome virus is a contagious virus. The PRRSV negative pigs exposed the virus by oronasal, intramuscular, intravenous, intrauterine and intraperitoneal routes can become infected (Albina et al., 1994; Christianson et al., 1992; Lager et al., 1997). Oronasal exposure is considered to be a natural route of infection. Pigs can be infected by a small amount of the virus. A small amount of the virus as low as 10 virus particles can cause infection (Zimmerman et al., 1997b). Pigs have also been infected by intramuscular inoculation with as few as 10 virus particles (Yoon et al., 1999). The dosage causing infection by other routes has not been determined. A contaminated needle is considered as a potential source of spreading

virus. Otake et al. (2001) found that virus was transmited from PRRSV positive pigs to PRRSV negative pigs by the use of the same needle.

Direct contact seems to be the primary mode of transmission. Naïve pigs were viremic and seroconverted when commingled with PRRSV infected pigs (Wills et al., 1997a, 2000). In endemically infected herds, older viremic pigs can shed the virus to younger pigs (Stevenson et al., 1993). Vertical transmission was demonstrated experimentally when a sow inoculated with PRRSV 99 days earlier was housed with 3 seronegative finisher pigs (Zimmerman et al., 1992). The transmission was demonstrated when in-contact pigs exhibited seroconversion. In another study, sentinel pigs exhibited seroconversion after they were housed in direct contact with pigs experimentally infected with PRRSV 56 days earlier (Terpstra et al., 1992). More recently, two groups of pigs were infected experimentally with two virulent PRRSV isolates and sentinel pigs were commingled sequentially with the infected pigs (Wills et al., 2000). The sentinel pigs did not seroconvert after they were placed in direct contact with the infected pigs 67-69 days after infection. On the other hand, pigs placed in contact prior to day 63 seroconverted. In another experiment, pigs were infected with PRRSV and sentinel pigs were sequentially placed in contact with them. Two of the 4 sentinel pigs placed in contact with the infected pigs 24 days after infection did not seroconvert (Yoon et al., 1993). The failure of these pigs to become infected suggested that the amount of virus in the room decreased over time, or the pigs in the last group might have been resistant because they were older when placed in contact. The seronegative pigs that were placed in indirect contact or separated from the infected pigs by an extended distance did not seroconvert (Wills et al., 1997a).

Seropositive boars infected naturally or by vaccination are considered to be reservoirs of infection (Christopher-Hennings et al., 1995a; Swenson et al., 1994). Viruses can replicate in the testicular cells of boars and then be shed via semen up to 92 days p.i (Christopher-Hennings et al., 1995b). It has been shown that virus shed from semen of boars is infectious (Swenson et al., 1994). Non-infected pigs become infected when they are inoculated with contaminated semen. Gilts or sows can become infected when they are inseminated naturally by infected boars or artificially by contaminated semen. Frozen semen containing the virus is considered to be a potential source of virus. Yaeger et al. (1993) demonstrated that gilts seroconverted when they were inseminated with semen from PRRSV infected boars.

Pigs vaccinated with PRRSV MLV (RespPRRS®, Boehringer Ingelheim Vetmedica, Inc. and PRIME PAC™, Schering-Plough) shed vaccine virus. The vaccine virus was recovered from serum and semen of boars (Christopher-Hennings et al., 1997; Torrison et al., 1996). Torrison et al. (1996) found that sentinel pigs seroconverted after commingling with MLV vaccinated pigs.

Transplacental infection has been demonstrated in pregnant pigs exposed to North American or European isolates at various stages of gestation (Christianson et al., 1992, 1993; Kranker et al., 1998; Lager and Mengeling, 1994, 1995; Prieto et al., 1996). Virus was isolated from liveborn as well as stillborn piglets, but was not isolated from mummified fetuses (Christianson et al., 1993). Transplacental transmission did not occur in pregnant pigs exposed to the virus during the first 7 days of gestation (Prieto et al., 1996). During early gestation, fetuses may be more resistant to PRRSV (Lager and Mengeling, 1995). It is less likely that fetal death occurs during the early phase of gestation (Mengeling et al., 1994). The prevalence of transplacental transmission was 0 or low when pregnant pigs were inoculated with virus at approximately 45-50 days of gestation (Christianson et al., 1992, 1993; Kranker et al., 1998). Greater susceptibility occurs during late mid-gestation, as indicated by recovery of the virus from fetuses of dams exposed to the virus on days 50-72 of gestation (Christianson et al., 1992, 1993; Kranker et al., 1998). The prevalence of transplacental transmission is higher as the late phase of gestation approaches. Exposure of pregnant pigs at approximately 90 days of gestation results in the highest prevalence of transplacental transmission (Andreyev et al., 1997; Mengeling et al., 1994, 1996, 1999a).

The mechanism of transplacental infection still needs to be elucidated. However, it has been suggested that infected macrophages may cross the placenta to infect the fetus and late gestational fetuses are more susceptible to virus infection (Terpstra et al., 1991). The detection of anti-PRRSV specific antibodies in the sera of presuckling pigs farrowed from pigs exposed to the virus on 85-90 days of gestation might mean that the fetuses were infected within 10 days after maternal exposure (Mengeling et al., 1996).

Pigs 2 to 3 week-old with maternal immunity are more likely protected from infection than pigs 6 to 9 week-old (Chung et al., 1997). Gramer et al. (1998) demonstrated that piglets born from previously infected sows could be used to produce PRRSV negative isowean pigs. In addition, Donadeu et al. (1997) reported that hysterectomy-derived pigs and fostered with PRRSV negative sows, medicated early weaning (MEW), and

isowean had a high rate of success in producing PRRSV negative piglets from endemically PRRSV infected herds.

The PRRSV causes disease only in pigs. However, Muscovy ducks, Mallard ducks, guinea fowl, and chickens have been reported as the potential sources of virus spreading (Zimmerman et al., 1997a). Virus did not replicate in these animals. Virus was isolated from pooled feces of mallard ducks that were exposed to virus up to 25 days p.i. Moreover, pigs intranasally exposed to PRRSV isolated from mallard feces became viremic, seroconverted as determined by ELISA, and transmitted the virus to sentinel swine. Mice and rats are not infected by PRRSV (Hooper et al., 1994).

Diagnosis of PRRSV

Clinical observation

Various diagnostic methods have been described for PRRSV detection. The diagnosis of PRRSV infection is based on the presence of clinical signs, gross and histopathological lesions, PRRSV specific antigen and PRRSV specific antibody. Clinical signs consisting of late-term abortions, increased numbers of stillborn, mummified and weak fetuses, and increased preweaning mortality are common in herds experiencing PRRSV infection (Christianson and Joo, 1994; Mengeling and Lager, 2000). Many PRRSV infections are clinically mild or inapparent. A diagnosis based on clinical signs only is difficult to achieve since there is a wide variation among herds (Christianson and Joo, 1994). The variation from pig to pig and herd to herd including secondary bacterial infections may confound the typical PRRSV clinical signs.

Gross and microscopic lesions

Gross lesions are generally confined to the lungs and lymph nodes. Most gross lung lesions are characterized by a diffuse consolidation of the lung producing a mottled and tan coloration with cranioventral distribution. A generalized lymphadenopathy is observed in cervical, tracheobronchial, mesenteric and inguinal areas (Halbur et al., 1996b, 1996a; Rossow et al., 1994a). Gross lesions are not always observed in PRRSV infection. Thus, histological examination is needed to further confirm findings from gross lesions. Interstitial pneumonia characterized by alveolar septa thickening with infiltration of mononuclear cells is observed consistently. The second most affected organs are the lymph nodes. The lymph nodes of infected pigs are characterized by germinal hypertrophy and hyperplasia, and germinal center necrosis. Other histopathological

lesions including rhinitis, nonsuppurative encephalitis and perivascular mononuclear myocarditis may help in differentiation but may not be as consistent a finding as interstitial pneumonia (Halbur et al., 1996a; Rossow et al., 1994a).

Virus isolation

Porcine reproductive and respiratory syndrome virus can be detected by virus isolation (VI). Pulmonary alveolar macrophages (PAMs) appear to be the most suitable cells for VI (Bautista et al., 1993). They should be harvested from pigs younger than 6-8 weeks of age. Different batches of PAMs vary in susceptibility to the virus, so each batch must be tested before use. It makes the assay laborious (Botner, 1997). The commercial cell line, MARC-145, is routinely used for VI (Kim et al., 1993). The virus can also be isolated in some commercial cell lines including CL2621, CRL 11171, and MA-104 (Benfield et al., 1992; Kim et al., 1993; Meng et al., 1996). Co-cultivation of PAMs and MARC-145 is more sensitive than using either PAMs or MARC-145 alone (Mengeling et al., 1995). Virus was isolated from lung lavage samples of infected animals up to 63 days p.i. using this co-cultivation method. Attempts to use other cell lines such as canine kidney, feline kidney, rabbit kidney and porcine kidney cell have failed to propagate PRRSV (Benfield et al., 1992; Mardassi et al., 1994; Wensvoort et al., 1991). The sensitivity of VI can be enhanced by adding serum containing PRRSV antibodies (Christianson et al., 1993).

Porcine reproductive and respiratory syndrome virus can be isolated from serum and several tissues including lungs, lymphoid tissues, tonsils, spleens of infected pigs (Halbur et al., 1996a, 1996b; Rossow et al., 1994a, 1998). Autolyzed and mummified fetuses are not suitable for VI (Christianson et al., 1993). Attempts to isolate the virus from samples of acutely infected pigs were more successful than from chronically infected pigs. During the early phase of infection, PRRSV can be isolated from serum, saliva, urine and lung lavage (Mengeling et al., 1995; Wills et al., 1997b). Attempts to isolate virus from feces and conjunctival swabs have not been successful (Wills et al., 1997b). Virus isolation from lung lavage collected post mortem is more sensitive than from other tissues. Virus can be isolated up to 63 days p.i. (Mengeling et al., 1995, 1999b). Virus can be isolated from oropharyngeal scrapings through 157 days p.i. (Wills et al., 1997c).

Reverse transcriptase-polymerase chain reaction assays (RT-PCR)

In recent years, a number of investigators have developed reverse transcriptase-polymerase chain reaction assays (RT-PCR) for the detection of PRRSV nucleic acid in serum and other diagnostic samples. The RT-PCR test is slightly more sensitive than virus isolation for detection of virus in serum (Christopher-Hennings et al., 1995b; Spagnuolo-Weaver et al., 1998). The test is able to detect PRRS virus nucleic acid in serum samples as early as 24 hours after infection. Primers specific for ORF 7 are most commonly used in the PCR diagnosis. By amplifying the specific ORF 7 region, RT-PCR can detect virus at concentrations as low as 10 virions per ml (Christopher-Hennings et al., 1995b). Primers designed for ORFs 4, 5 and 6 amplification were also evaluated for potential use; however, the specificity decreased when using primers designed for these ORFs (Guarino et al., 1999). A decrease in the specificity was due to the genomic variability among PRRSV isolates.

Detection of PRRSV antigen in tissue section

The direct fluorescent antibody staining (FA) test, immunohistochemistry (IHC) and *in situ* hybridization (ISH) can be used to detect the presence of PRRSV antigen in tissue sections. The FA is done by staining tissue sections with an anti-PRRSV Mab fluorescein conjugate. The test is then read using a flurescent microscope. The IHC is used to detect PRRSV antigens in formalin-fixed tissues. It is more sensitive than the FA test. Two types of IHC utilizing immunoperoxidase (Halbur et al., 1995a) and immunogold silver staining were developed (Larochelle et al., 1994; Magar et al., 1993). Immunogold silver staining was reported to be more sensitive than immunoperoxidase staining in detecting PRRSV antigen in formalin-fixed tissues. *In situ* hybridization was developed for the detection of PRRSV in cell cultures and in formalin-fixed paraffin-embedded tissue (Larochelle et al., 1996; Sur et al., 1996). The ISH technique detects the distribution of PRRSV RNA in various tissues. During the early phase of infection, ISH detected greater amounts of PRRSV RNA in lungs than in lymphoid tissue. The sensitivities of ISH and IHC were equivalent during the early phase of infection. In contrast, ISH was more sensitive than IHC during the late phase of infection (Sur et al., 1996).

Serological assays

Serological tests represent powerful tools for the detection of PRRSV infection. The presence of anti-PRRSV specific antibodies in pigs that are not vaccinated with PRRSV vaccine indicates previous exposure to

the virus. Several serological methods including the indirect immunofluorescence assay (IFA), the immunoperoxidase monolayer assay (IPMA), the ELISA, and serum neutralization (SN) test have been developed (Botner, 1997; Cho et al., 1996; Sorensen et al., 1998; Yoon et al., 1992, 1995). The immunoperoxidase monolayer assay was the first serological test developed for the detection of PRRSV specific antibody (Wensvoort et al., 1991). Initially, the assay was performed using PAMs. Later on, the test was developed to use either PAM or continuous cell lines such as MARC-145 or CL 2621. Meanwhile, IFA that is equivalent to IPMA was developed in the U.S.A. (Yoon et al., 1992). Both tests can detect PRRSV specific antibody as early as 7 days p.i. (Yoon et al., 1992, 1995). The sensitivity and specificity of IFA were 81.7% to 98.5% and 97.7% to 100%, respectively (Cho et al., 1997b). The sensitivity of IPMA was 99.8% that was higher than IFA.

The ELISA can detect PRRSV specific antibody as early as 9-13 days p.i (Yoon et al., 1995). The specificity of the ELISA is similar to that of the IFA and IPMA, but its sensitivity is higher (Cho et al., 1996, 1997a, 1997b). Its sensitivity approaches 99.6% (Cho et al., 1997b). An ELISA kit manufactured by IDEXX Inc. (HerdCheck® PRRSV) has been widely used. The kit recognizes antibodies in pigs exposed to both European and U.S. isolates because it utilizes antigen derived from both isolates. Competitive ELISA using the N protein as antigens was developed (Dea et al., 2000). However, the sensitivity of the competitive ELISA is lower than that of the commercial HerdCheck® ELISA. Testing 2 sets of serm samples, the competitive ELISA was able to detect the anti-PRRSV specific antibodies in 86.7% and 92.6% of pigs known to be seropositive, while the HerdCheck® ELISA detected the anti-PRRSV specific antibodies in 100% and 96.2% of pigs known to be seropositive pigs, respectively.

The SN test is performed in microtiter plates using either CL2621or MARC-145 cell cultures (Yoon et al., 1995). The SN test cannot be performed on PAMs since the presence of Fc receptors on the surface of macrophages may uptake the virus-antibody complex and support virus replication. Specific SN antibody to PRRSV appears to develop slower than other viruses. The SN antibody specific to PRRSV is first detected in infected pigs at 28-35 days p.i. The SN antibody remains up to 5-7 months p.i (Nelson et al., 1994; Osorio, 1999). The standard SN assay was modified by the addition of 20% fresh seronegative swine serum (Yoon et al., 1994). This modification increases the sensitivity of the test and detects SN antibody as early as 11 days p.i.

The addition of 20% fresh guinea pig serum also increases the test sensitivity to detect specific SN antibody as early as 8 days p.i. (Takikawa et al., 1997).

Swine bioassay

It is difficult to isolate the virus from semen because semen is very cytotoxic for cell cultures. Although semen can be diluted, the sensitivity of test would decrease. A swine bioassay was developed to detect virus in semen and to determine if virus in semen that is detected by RT-PCR is infectious (Swenson et al., 1994). The swine bioassay was done by intraperitoneal inoculation of fresh samples into four to eight week-old pigs. Subsequent seroconversion for PRRSV specific antibodies or virus isolation in inoculated pigs indicated semen was contaminated with infectious virus. The swine bioassay has shown a good correlation with RT-PCR in the detection of infected animals (Swenson et al., 1994).

Immune response

Following infection, PRRSV infected pigs develop both humoral and cell-mediated immune responses. The humoral immune response is detected by several serological assays, including IFA, IPMA, ELISA, and SN (Albina et al., 1998; Loemba et al., 1996; Nelson et al., 1994; Vezina et al., 1996; Yoon et al., 1995). The cell-mediated immune (CMI) response is detected by lymphocyte proliferation assay and the number of PRRSV specific interferon-gamma (IFN-γ) producing cells (Bautista and Molitor, 1997; Meier et al., 2000b; Vezina et al., 1996).

Humoral immune response

Specific anti-PRRSV IgM antibodies (IgM) as measured by IFA are first detected as early as 5-7 days p.i (Park et al., 1995). Specific IgM reaches its highest level at 9-10 days p.i. and decreases from its peak level at 14-21days p.i. to undetectable levels by 35-42 days p.i.

Specific PRRSV IgG antibodies are first detected by the IPMA at 5-9 days post inoculation and reach maximum levels by day 28-42. Specific antibodies detected by IFA, ELISA and SN appeared on 9-11, 9-13 and 9-28 days p.i., respectively (Yoon et al., 1995). The antibody titers detected by these tests reached maximum levels by 28-35, 35-42, and 77-84 days p.i., respectively. Once the highest level was reached, the antibody titer assayed by all four serological tests decreased thereafter. Statistical calculation by logistic regression analysis

estimated that antibody titers detected by ELISA, IFA, IPMA and SN would have disappeared by approximately 137, 158, 324 and 356 days p.i., respectively.

The first initiation of PRRSV antibody response is directed against the N protein. The N protein is considered to be an immuno-dominant protein due to its strong signal observed by Western immunoblotting during the first few weeks after exposure (Loemba et al., 1996). An association between the N protein and viral neutralization has not been demonstrated (Loemba et al., 1996; Nelson et al., 1994; Vezina et al., 1996; Yoon et al., 1992). Studies of antibody response towards the structural proteins N, M, and glycoprotein 5 (GP5) by Western immunoblotting have yielded variable results (Loemba et al., 1996; Nelson et al., 1994; Yoon et al., 1995). Antibody response detected by 7 days p.i. was directed against the GP 5. Antibody response detected by 14 days p.i. was directed against the N and M protein (Loemba et al., 1996). However, other studies revealed that seroreactivity to GP5 is generally observed by 21-28 days p.i. which coincides with the presence of SN antibodies (Nelson et al., 1994; Yoon et al., 1995).

Cell-mediated immune response

The CMI response in pigs following PRRSV inoculation is first detected at 28 days p.i. and persists for more than 3 months as detected by lymphoproliferation assays (Bautista and Molitor, 1997; Bautista et al., 1999; Lopez Fuertes et al., 1999; Vezina et al., 1996) The specific CMI response approaches the maximum level by 49 days p.i. and decreases by 77 days p.i. Anamnestic response toward T-cell proliferation increased in magnitude when it was induced by a second virus exposure. However, the anamnestic response lasts only 2 to 3 weeks (Bautista and Molitor, 1997). The lymphocyte proliferations were antigen-specific and dose-dependent (Bautista and Molitor, 1997). Recently, CMI was also studied by the enzyme-linked immunospot assay (ELISpot) (Meier et al., 2000a, 2000b). This assay is capable of enumerating interferon gamma (IFN-γ) producing T-cells. The number of IFN-γ producing T-cells is directly proportional to the CMI (Zuckermann et al., 1998). The PRRSV specific interferon-gamma producing T-cells are not detected until 8-9 weeks p.i (Meier et al., 2000a, 2000b).

The individual structural proteins N, M, GP2, GP4 and GP5 were experimentally evaluated for their ability to elicit specific T-cell responses by lymphoproliferation assays (Bautista et al., 1999). Individual proteins were obtained from a vaccinia virus expression system. Low proliferative responses were also observed

in pigs immunized with GP5, GP2 and GP4. Greater proliferative responses were observed in pigs immunized with the M protein. Immunization with nucleocapsid protein induced the lowest lymphocyte cell proliferative response.

Significant enhancement of lymphoproliferation assays start at 7 days p.i. and then continue to increase significantly until approximately 14 days p.i. (Vezina et al., 1996). The lymphoproliferation to mitogens indicates that, during the early phase of infection, PRRSV infection appears to diminish proliferative activity of blood lymphocytes (Vezina et al., 1996). However, the diminution of specific immune reactivity appears to be overcome later by the host because antibody and cellular immune responses occur simultaneously.

During early PRRSV infection, the total leukocyte population in the broncho alveolar lavage (BAL) fluid is increased. There is strong evidence suggesting that this increase is due to the influx of both natural killer cells and cytotoxic T-cells into the lung (Samsom et al., 2000). The total number of recovered BAL cells increases 10 times between 10 and 21 days p.i. Thereafter, the number of cells decreases. In contrast, another study by Labarque et al. (2000) suggested that an increase of BAL cells following the PRRSV infection was due to the influx of monocytes and macrophages.

In the circulation, PRRSV infection affects certain types of lymphocyte cells (Kawashima et al., 1999; Lopez Fuertes et al., 1999; Shimizu et al., 1996). The total number of lymphocytes markedly decreases after infection (Kawashima et al., 1999). The decline of $CD4^+$ T-cells is observed until 14 days p.i. The number of $CD4^+$ T-cells returns to normal levels thereafter. The number of $CD8^+$ T-cells decreases slightly until 3 days p.i. and then increases to maximum levels by days 28 to 35 p.i. The ratio of $CD4^+/CD8^+$ T-cells decreases during acute and chronic phases of PRRSV infection. A decrease during acute infection is due to a decrease of $CD4^+$ T cell (Shimizu et al., 1996). A decrease during chronic phase of infection is due to an increase of $CD8^+$ T cell. Since $CD4^+$ and $CD8^+$ T-cells are not susceptible to PRRSV, it has been suggested that the mechanism involved in the alteration of T-cell population is regulated by Th1-cytokines (Lopez Fuertes et al., 1999).

Protective immunity

The development of protective immunity to PRRSV infection has been studied in both naturally and experimentally infected animals. Protective immunity was first detected in field observations when PRRSV infected sows delivered normal litter sizes following rebreeding despite virus circulation on the farm (Albina et

al., 1994; Bilodeau et al., 1994). Moreover, previous exposure to PRRSV can prevent the development of clinical signs and reduce the magnitude of virus shedding after subsequent rechallenge with PRRSV (Shibata et al., 2000). These observations suggest that PRRSV infected pigs develop protective immunity following infection.

Lager et al. (1997, 1999) demonstrated complete protection following rechallenge with homologus PRRSV. In the study, pregnant gilts were exposed to the virus on either day 1 or day 30 of gestation. Infected gilts were then rechallenged with homologous PRRSV on day 90 of gestation. Gilts in the challenge control group were exposed to the virus only at day 90 of gestation. Transplacental infection was used to measure protective immunity. Transplacental infection and reproductive losses were not observed in pigs that were infected and rechallenged with the same strain of PRRSV. In contrast, transplacental infection and reproductive losses occurred in pigs that were exposed to PRRSV only on day 90 of gestaion. This finding suggested that gilts developed protective immunity against the homologous strain. The duration of protective immunity lasted up to 604 days p.i (Lager et al., 1997b). Complete homologous protection of the reproductive tract was also observed in pigs vaccinated with MLV and rechallenged with the same vaccine virus on 120-140 days after vaccination (Gorcyca et al., 1997; Hesse et al., 1997).

Another study demonstrated that reinfection with a homologous strain of PRRSV could occur (Shibata et al., 2000). Pigs were challenged on day 0 and then rechallenged with a homologous strain on 77 days p.i. Although the pigs did not develop clinical signs, viremia was detected 3 days after rechallenge. However, the viremia was low in magnitude and short in duration, and no virus was isolated from any tissue after death.

Heterologous protection is incomplete and may have a shorter duration than homologous protection (Lager et al., 1999). In a study by Lager et al. (1999), gilts were inoculated with virulent PRRSV strain NADC-8 prior to breeding, and rechallenged with antigenically distinct LV isolate or homologous PRRSV strain NADC-8 from 90 to 170 days post inoculation. Specimens from gilts and fetuses were tested for virus. Lack of virus in gilts was considered to indicate protective immunity for the dam. Absence of virus in fetuses indicated protection of gilts from reproductive losses. Absence of virus in both groups indicated complete protection. Complete protection was observed among pigs rechallenged with the homologous strain. Virus was not isolated from any gilt or piglet. In contrast, gilts rechallenged with the heterologous strain were only partially protected.

Virus was detected in gilts necropsied 134 to 170 days after rechallenge and in one litter necropsied 170 days after rechallenge.

A study by Benson et al. (2000) demonstrated that heterologous protection might be related to the challenge dose. In the study, gilts were vaccinated with PRRSV MLV (RespPRRS/Repro®, Boehringer Ingelheim Vetmedica, Inc.) prior to breeding and subsequently rechallenged with PRRSV strain NADC-8 at three different doses on day 90 of gestation. Transplacental infection did not occur in gilts rechallenged with a low dose of virus (10^2 $TCID_{50}$/ml), whereas it was exhibited in gilts rechallenged with 10^4TCID_{50}/ml and 10^6 $TCID_{50}$/ml of the virus. These observations suggested that protection is dose-dependent.

Vaccines

Traditional vaccines

Several PRRSV vaccines are available on the market, including both MLV and KV. Initially, 3 MLVs were marketed, including RespPRRS/Repro® from Boehringer Ingelheim Vetmedica, Inc., PRIME PAC™ from Schering-Plough Animal Health Corporation, and Suvaxn® from Fort Dodge Animal Health, Inc. Currently, only RespPRRS/Repro® is commercially available. PRRomiSe® from Intervet Corporation is a commercial KV. Homolgous autogenous killed vaccines are also available and any USDA-licensed biologic company can manufacture them upon request.

The MLV RespPRRS/Repro® was first marketed in 1994 and has been on the market since then. This MLV is derived from the PRRSV parent strain ATCC VR-2332. The vaccine virus differs from its parent strain at amino acid residues 13 and 151 of ORF5. The parent strain has an arginine at both positions, but the vaccine virus has a glutamine and a glycine at residue 13 and 151, respectively (Wesley et al., 1999). The recommendation is for use only in 3-18 week-old pigs and non-pregnant females. Vaccinated gilts were not viremic following homologous or heterologous challenge. Vaccinated and challenged gilts also farrowed more live healthy piglets than non-vaccinated and challenged gilts (Gorcyca et al., 1997).

The MLV PRIME PAC™ was first marketed in 1996 and derived from virulent PRRSV strain Neb-1. It is used to reduce reproductive disease in female breeding age pigs and respiratory disease in pigs 3-4 weeks of age or older. The vaccine is recommended for use in pigs 3-4 weeks of age or older, and for female pigs up to 6 weeks prior to each breeding. The vaccine was demonstrated to significantly reduce the severity of disease

following heterologous challenge at 85 days of gestation. Comparative studies have shown a decrease in piglet mortality and clinical abnormalities associated with PRRSV infection such as mummies and stillbirths (Hesse et al., 1997). Vaccinated gilts were not viremic following homologous or heterologous challenge. Vaccines were not recommended for use in gestational pigs (Lager et al., 1999b). The PRIME PAC™ vaccine was removed from the market in 2000.

The disadvantage of using MLV vaccine is that vaccine viruses are shed from vaccinated pigs for an extended period of time (Christopher-Hennings et al., 1997; Swenson et al., 1995a; Yaeger et al., 1993). Torrison et al. (1996) reported the transmission of vaccine virus from MLV vaccinated pigs to naïve pigs up to 36 days after vaccination. The use of MLV in boars resulted in vaccine virus shedding in semen as well as a reduction in semen quality (Christopher-Hennings et al., 1997). Virus was detected in semen of vaccinated boars at least 92 days after vaccination.

The KV PRRomiSe® is manufactured by Bayer Corporation, and is currently marketed by Intervet, Inc. It is recommended for use in dams 5-8 weeks after breeding, followed by a second dose 14-28 days later. Vaccinated gilts were non-viremic following homologus challenge (Christensen et al., 1998). The number of viable pigs born to vaccinated gilts was significantly greater than those of pigs born from non-vaccinated gilts. In another study, vaccination with inactivated virus resulted in protection from homologous challenge. The vaccinated sows farrowed 70% live and healthy piglets, whereas only 10% of the piglets from non-vaccinated sows were born alive and healthy (Plana-Duran et al., 1997a).

New generation of vaccines

The PRRSV proteins expressed in insect cells using the baculovirus system have been evaluated as potential subunit vaccines (Kreutz and Mengeling, 1997; Plana-Duran et al., 1997b). Open reading frames 2-7 were inserted into recombinant baculoviruses. However, only ORFs 2, 3, 5 and 7 representing GP 2, 3, 5 and the N proteins, respectively were successflully expressed (Plana-Duran et al., 1997b). A similar study reported that only ORFs 5, 6 and 7 were successfully expressed (Kreutz and Mengeling, 1997). To analyze their ability to confer protection, Plana-Duran et al. (1997b) immunized sows with recombinant baculoviruses expressing the products of ORFs 2, 3, 5 and 7. Protection was observed in sows immunized with recombinant baculoviruses expressing the products of ORFs 3 and 5. These sows successfully weaned 68.5% and 50% of

healthy piglets following challenge. In contrast, non-immunized control sows and sows immunized with recombinant baculoviruses expressing the products of ORFs 2 weaned only 12.6% and 16.6% of healthy pigs (Plana-Duran et al., 1997b). These results suggested that ORFs 3 and 5 are involved in the induction of protective immunity (Plana-Duran et al., 1997b).

The genome of PRRSV is comprised of 8 ORFs, in which 6 ORFs encode the viral structural proteins. The genomic regions encoding for ORFs 4-7 were studied to determine their potential use in DNA vaccines by cloning in a mammalian expression vector (Kwang et al., 1999). Pigs were injected intramuscularly with DNA plasmids containing ORFs 4-7 every 3 weeks. Cell-mediated immunity as measured by the number of PRRSV specific IFN-γ producing cells and the lymphoproliferation assay was detected in all pigs immunized with DNA plasmids containing ORFs 4-7. Specific PRRSV neutralizing antibodies were detected in pigs immunized with DNA plasmids containing ORFs 4 and 5. This observation suggests that the targets of SN antibodies are epitopes of proteins encoded by ORFs 4 and 5 of PRRSV. Yang et al. (2000) reported that Mabs representing proteins encoded by ORFs 5 and 6 have neutralizing activity.

Immunization with a plasmid DNA encoding the GP5 of PRRSV protected pigs from developing lung lesions and viremia (Pirzadeh and Dea, 1998). Immunized pigs also developed PRRSV specific SN antibodies. Serum neutralizing antibodies were not detected until 2-3 weeks after the second immunization. In contrast, immunization with the recombinant protein of GP5 expressed by *E.coli* did not induce an immune response. These observations suggested that the target of SN antibodies is epitopes of proteins encoded by ORF 5 of PRRSV.

Since a degree of antigenic diversity has been observed among PRRSV isolates, a vaccine based on a single strain may not be effective against an antigenically distinct PRRSV strain (Meng, 2000). Meng (2000) suggested that the effectiveness of PRRSV vaccine may vary depending on the antigenic relatedness of the isolates to which pigs are exposed. Therefore, Meng (2000) suggested that an attenuated multivalent vaccine comprised of multiple antigenically distinct strains of PRRSV would be effective.

Elimination and control

Several management protocols have been used to eliminate PRRSV from swine herds. These protocols include total depopulation and repopulation with PRRSV-negative replacements, partial depopulation such as

nursery depopulation, test and removal, isowean, whole herd vaccination, and temporary herd closure with the introduction of PRRSV-negative replacements (Andreasen et al., 1998; Dee and Molitor, 1998; Donadeu et al., 1997; Hassing et al., 2000; Philips et al., 2000; Torremorell et al., 2000). These management protocols have resulted in varying degrees of success. Prior to the implementation of any strategy, several factors should be considered, such as the existence of subclinical disease in the herd, strain variation, the level of herd immunity and the persistence of the virus in infected populations. In addition, strict biosecurity measures should be applied to all herds when considering virus elimination. Introduction of virus into a herd by any means, including mechanical vectors or PRRSV-infected replacement pigs, should be avoided. Furthermore, only semen free of PRRSV should be used.

Total depopulation and partial depopulation have been evaluated as methods to eliminate PRRSV from herds (Andreasen et al., 1998; Hassing et al., 2000). Total depopulation has resulted in successful elimination of infectious agents not only for PRRSV, but also for infectious agents such as pseudorabies virus (Thawley and Morrison, 1988). Total depopulation requires the elimination of an entire herd, followed by disinfection of the empty units which are left unoccupied for 14 days. Repopulation requires using PRRSV-negative replacements. Prior to introduction into the breeding unit, PRRSV-negative replacements must be held in an isolation unit for at least 21 days to ensure they are free of PRRSV. Although total depopulation seems to be the best approach, it is expensive to conduct.

Partial depopulation has also been carried out. This protocol is preferred to total depopulation because some pigs can remain within a herd to avoid production losses (Hassing et al., 2000). Partial depopulation is accomplished by removing the potential source of virus spread, such as dams less than 10 months-old, nursery or finisher-age pigs. Then the empty rooms and buildings are disinfected and left unoccupied for 14 days. The essential requirement for partial depopulation is that the immunity of the breeding unit must be stable. There should be no evidence of active virus circulating on the farm. Immunological stability refers to the immune status of the herd in which, at most, 10% of the sows have an IPMA titer of 1,250 and no sows have a titer higher than 6,250 (Hassing et al., 2000). The presence of sows in a herd with titers that exceed these levels is an indication of an acute phase of infection (Botner, 1997).

Nursery depopulation has also been considered as a partial depopulation method to eliminate PRRSV (Dee and Joo, 1994; Dee et al., 1993, 1997a, 1997b, 1999). Nursery depopulation is based on observational data that indicates that transmission of PRRSV occurs between older animals and recently weaned pigs (Dee and Joo, 1994). The removal of nursery pigs interrupts the circulation of virus and decreases the likelihood of transmission from older to younger pigs. Nursery depopulation is accomplished by removing nursery pigs from the site, disinfecting the empty facilities, and leaving them empty for 14 days. This method has been reported to be effective on farms with sow populations less than 1,000 (Dee and Joo, 1994).

Tubbs and Thompson (1999) successfully eliminated PRRSV from a two-site isowean herd. It was concluded that nursery depopulation and partial depopulation of finishers were essential to PRRSV elimination. The nursery and finisher buildings in closest proximity to the nursery remained empty for several months, which allowed time for virus excretion to cease in older finishers that were housed in the more distant buildings. In addition, the movement of both humans and pigs was minimized to decrease the likelihood of virus spread.

Plomgaard (1998) was able to simultaneously eliminate PRRSV and *Mycoplasma hyopneumoniae* from a three-site farm. All females 10-months-old or less were removed from site 1 and the herd was closed to replacements for 4 months. All nursery pigs were depopulated. Off-site breeding was conducted to supply PRRSV-negative replacements to the system. Sows in the breeding unit were tested by ELISA. The non-immune pigs were removed from the system. All piglets were weaned off-site at 21 days.

The test and removal protocol has been carried out for PRRSV elimination (Dee and Molitor, 1998; Dee et al., 2000a, 2000b, 2001). The principle concept of the test and removal protocol is detection and removal of infected pigs, including persistently-infected pigs from the breeding unit. The test and removal protocol involves sampling the entire breeding herd in one day, and testing sera by IFA, ELISA and PCR to detect previously exposed and/or infected animals. Pigs are removed if they are positive by either test. Pigs with an ELISA titer of less than one but negative by PCR are retained and retested one month later. The weakness of the test and removal protocol is the potential risk of failure to detect persistently-infected animals using current diagnostic methods. A second concern is the high cost of the test and removal protocol. Another problem of this strategy is the current inability to verify whether sows with an ELISA titer of less than 1 are truly negative for virus.

Isowean is a management strategy in which piglets are weaned into an isolated site to separate them from all other age groups of swine. It is a practical method for excluding most pathogens from herds and reducing transmission of pathogens from older to younger pigs (Christianson et al., 1994; Harris, 1999). Donadeu et al. (1997) reported the success of isowean, MEW, and hysterectomy to obtain large numbers of PRRSV-negative piglets, in which 91, 92, and 94% of the batches weaned from PRRSV-positive sows were PRRSV-negative, respectively. These procedures were carried out with herds that had sow inventories less than 1,000. Gramer et al. (1998) also reported that multiple site isowean production consistently produced PRRSV-negative weaned pigs from PRRSV-positive sows. In the study, piglets were weaned at 18-23 days of age from PRRSV-positive sows to a clean nursery. After 50 days, the pigs were moved into a finisher unit, which was also managed as all-in/all-out. In some cases, when a high level of circulating virus is present in the herd, medicated early weaning (MEW) of pigs less than 8 days of age may be successful (Harris, 1999). The main factor influencing the success of infectious agent exclusion by isowean and MEW is the level of sow and piglet immunity (Harris, 1999).

Vaccination of entire herds with PRRSV MLV appears to increase the success rate of producing PRRSV-negative isowean pigs as well as eliminating PRRSV (Philips et al., 2000). This protocol consists of (a) stocking the sow unit with a 4-month supply of PRRSV-negative replacements; (b) closing the herd to new entries; and (c) vaccinating the entire herd as well as new replacements to maximize herd immunity (Philips et al., 2000). Prior to the entry of new replacements, the herd must be stabilized against active virus circulation. Piglets must be weaned off-site and the herd maintained under strict biosecurity measures. This protocol has resulted in PRRSV-negative isoweans with no evidence of active virus transmission.

Temporary herd closure to any replacement is another strategy for PRRSV elimination (Henry et al., 2000; Torremorell and Baker; 2000; Torremorell et al., 2000). Herd closure and restocking the sow unit only with PRRSV-seronegative replacements are the major features of this strategy (Torremorell and Baker, 2000). The rationale for temporary closure of the sow unit is based on several reports that virus shedding from individual pigs decreases over time. Individual pigs will eventually become free of PRRSV only if they are not re-exposed to the virus. When temporary herd closure of the sow herd is considered in an eradication plan, a decision must be made as to when PRRSV-negative replacements stock can again be introduced into the herd

with minimal risk of exposure to PRRSV. The length of temporary herd closure is not defined. However, 6 months of herd closure has been suggested (Henry et al., 2000; Torremorell and Baker, 2000). The introduction of PRRSV-negative replacements into the PRRSV-seropositive sow unit should only be made when there is no indication that PRRSV is circulating in the herd.

Acclimatization is accomplished by exposing replacement pigs to infectious agents present in a herd, to immunize the replacement pigs against existing infectious agents such as PRRSV, and allowing time for the replacement pigs to develop immunity and stop shedding virus. The acclimatization process can be accomplished by exposing replacement pigs to cull sows, or by feeding of PRRSV-contaminated tissues. The acclimatization period can range from 35 to 100 days

In PRRSV elimination, virus-negative replacement breeding stock is also the key to control and or elimination of the virus from infected herds. The introduction of PRRSV-positive replacement gilts into a herd is not recommended in PRRSV elimination. Precaution is advised because these pigs might be viremic upon arrival and a possible source of PRRSV to other pigs. Moreover, they could potentially introduce other strains of PRRSV that are antigenically different from existing strains. Intramolecular recombination between two distinct strains of wild-type PRRSV is possible (Yuan et al., 1999). Such recombination could potentially result in the emergence of a strain of increased virulence.

The PRRSV control strategy for each seropositive herd directs how the PRRSV-negative replacement breeding stock should be acclimatized prior to its introduction into the breeding herd. The seropositive herd can be categorized into PRRSV-positive herd with and without actively circulating virus. The introduction of PRRSV-negative replacement pigs into the seropositive herd could be accomplished by using the following steps.

Assuring the health status of PRRSV-negative pigs

Replacements should be obtained from herds free of PRRSV. The pigs should be placed in isolation for 21-28 days and tested serologically for PRRSV antibodies. This isolation period is necessary to avoid receiving pigs from a herd in whih pigs might be incubating the virus. It is also a safeguard against contamination during transport. It is advised that all stock be held in isolation for 21-28 days prior to the

acclimatization procedure and tested serologically to assure PRRSV-negative status. This isolation period ensures time to monitor the replacements for clinical signs of PRRS.

Introduction of PRRSV-negative pigs into a seropositive herd with no circulating virus

As indicated previously, a herd seropositive for PRRSV will become negative for the virus if the following criteria are met: (1) growing pigs are removed from contact with the sow herd, as in a one site farm; (2) no pigs recently infected with PRRSV are used as replacements for 4 months (production loss can be avoided by breeding PRRSV-negative replacements off-site); and (3) modified live vaccines are not used in the breeding herd or in growing pigs. A straightforward method to determine if a herd is free of the PRRSV is accomplished by introducing virus-free replacements and monitoring them serologically. If the replacements seroconvert, they should be removed. The three listed criteria should be satisfied prior to the introduction of PRRSV-negative replacements.

Introduction of PRRSV-negative pigs into a seropositive herd with circulating virus present

If a herd seropositive for PRRSV also has a high level of PRRSV, then one can anticipate persistent virus circulation in the herd if PRRSV-negative replacement pigs are introduced directly into the herd without appropriate acclimatization. Thus, the introduction of PRRSV-negative pigs is similar to the method used to eliminate transmissible gastroenteritis virus (TGEV) (Harris and Wiseman, 1989; Harris et al., 1987, 1999). In an acute TGEV outbreak, pigs at all age were fed with TGEV-contaminated tissued collected from TGEV-infected pigs. The purpose was to immunize pigs against TGEV. Simultaneously, an adequate supply of replacement pigs for restocking for 6 months were brought in and were exposed to TGEV as well. The herd was closed to any replacement pig for 6 months. The program resulted in elimination of TGEV from the herd. In a PRRSV-positive herd with circulating virus present, PRRSV-negative breeding stock of a wide variation in age is introduced to the breeding herd to provide replacements for the next 6 months of production. The entire herd was immunized by natural exposure to the virus. The herd was then closed to any replacement for the next 6 months. In addition to the natural exposure, Philips et al. (2000) recently used MLV in a mass vaccination scheme to immunize an entire herd, including PRRSV-negative replacement pigs. Thereafter, the herd was closed to replacements for 6 months. As the herd became immune, it started to produce PRRSV-negative pigs. The MLV would not be used again.

CHAPTER 3. A COMPARISON OF THE SHEDDING PATTERNS OF PIGS INFECTED WITH WILD-TYPE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) AND PIGS VACCINATED WITH A MODIFIED LIVE PRRSV VACCINE

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Abstract

This study was designed to determine if vaccine virus is shed for a shorter period of time than wild-type virus. A shorter virus-shedding period would decrease the acclimatization time of pigs before their introduction into a herd. Twenty-four 100-day-old crossbred pigs were randomly assigned in equal numbers (n=8) to 3 treatment groups: non-infected controls, vaccine virus, and wild-type virus. Pigs in the vaccine virus group were vaccinated intramuscularly with modified live virus vaccine (PRIME PAC™, Schering-Plough Animal Health) in accordance with the manufacturer's instructions. Pigs in the wild-type virus group were inoculated intramuscularly with 2 ml of PRRSV VR-2332 diluted to contain $10^{5.4}$ $TCID_{50}$/ml. Pigs in all groups were monitored for virus shedding over a 105-day period. Oropharyngeal scrapings were collected weekly, beginning at 49 days post infection (p.i.), and assayed for virus and virus RNA. On days 77 and 91, two groups of 2 sentinel pigs each were placed in contact with each group of pigs for 14-day periods. Virus was not isolated from any oropharyngeal scraping. In contrast, virus RNA was detected by PCR in oropharyngeal scrapings of 3 pigs infected with vaccine virus from 49 to 70 days p.i. and in oropharyngeal scrapings of 5 pigs infected with wild type virus from 49 to 105 days p.i. At necropsy, virus RNA was detected in tonsils of 3 pigs infected with vaccine virus and 2 pigs infected with wild-type virus. Sentinel pigs did not seroconvert even though they were housed with the principal pigs for 2 weeks. The detection of virus RNA in oropharyngeal scrapings and tonsils at necropsy suggests that virus remained in both groups of pigs but was not transmitted to contact sentinel pigs. Whether or not virus shedding can be reinitiated needs to be further evaluated.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the *Arteriviridae* (Cavanagh D. 1997). Since its first emergence in the late 1980s, PRRSV has caused a significant economic impact on the swine industry worldwide. Clinical signs of PRRSV infection include reproductive failure in dams, respiratory

disease in grower and finisher pigs, and increased pre-weaning mortality (Loula, 1991). An important characteristic of this virus is its persistent nature (Wills et al., 1997b). This persistent nature of PRRSV has interfered with current control and elimination programs.

Several management protocols have been used to control and eliminate PRRSV. These protocols include total depopulation followed by repopulation with PRRSV-free replacement pigs (Andreasen et al., 1998), partial herd depopulation such as the depopulation of entire nursery units to prevent the virus from being introduced into finisher units (Hassing et al., 2000), test and removal of infected pigs (Dee et al., 2001), and total herd vaccination (Philips et al., 2000). Temporary herd closure has also been used with success (Torremorell and Baker, 2000; Torremorell et al., 2000). This protocol involves closing the herd for an extended period of time to permit cessation or significant reduction of virus shedding, and restocking the breeding unit with PRRSV seronegative replacement pigs only. Replacement pigs enter the isolation and acclimatization process prior to introduction into a herd.

Acclimatization is the process of exposing replacement pigs to farm-specific strains of PRRSV, and allowing time for immunity to develop and virus shedding to stop. The acclimatization process is initiated by the feeding of tissues infected with farm-specific PRRSV or exposure to infected pigs. More recently, PRRSV-modified live virus vaccine (MLV) has been used to expose pigs to virus during acclimatization. The use of MLV would provide consistent exposure and, possibly, result in a shorter period of virus shedding. The duration of the acclimatization process that is used normally ranges from 35 to 100 days (Harris, 1999). This variability in duration could possibly be reduced if a protocol is developed that will allow the producer to predict the time when pigs develop immunity as well as when virus shedding is significantly reduced or stopped.

Several studies have demonstrated that infected pigs shed PRRSV for variable periods of time following infection. Wills et al. (1997a, 1997b) conducted two studies in which they infected pigs with PRRSV strain VR-2402. Virus shedding was monitored over a 124-day period in the first study and over a 213-day period in the second study. In both studies, virus shedding was monitored by collecting oropharyngeal scrapings starting at day 55, and assaying for virus and virus RNA. In the first study, virus was recovered from 2 of 4 pigs on days 55 and 84 (Wills et al., 1997a). No virus was isolated from any pig after day 84. In the second study,

virus was isolated from 2 of 4 pigs between days 55 and 84, and from a third pig on days 55 and 157 (Wills et al., 1997b). No virus was recovered after day 157.

Although virus can be detected in the secretions of infected pigs up to 157 days post infection (p.i.), it appears that virus transmission does not occur generally after day 70. Several researchers have investigated virus transmission in a similar manner, by placing groups of 1 to 2 sentinel pigs in contact with infected pigs at 2-week intervals. Wills et al. (2000) evaluated virus transmission over an 84-day period. In the study, virus transmission was observed between a group of 5 pigs infected with a PRRSV isolate with a restriction fragment length polymorphism pattern of 142, and sentinel pigs through day 62, but not thereafter. Terpstra et al. (1992) evaluated pigs infected with the LV isolate over a 70-day period and observed virus transmission between a group of infected pigs and sentinels through day 60. In contrast, Srinivasappa et al. (1999) evaluated transmission of the vaccine virus that was produced at the Fort Dodge Animal Laboratory over a 60-day period. They observed virus transmission between 5 vaccinated pigs and sentinel pigs though day 39, but not thereafter. These observations suggest that North American and European wild-type virus is commonly transmitted through 60-70 days p.i. The transmission period of vaccine virus might be significantly less than that of wild-type virus. However, additional simultaneous comparison studies of the shedding patterns of pigs infected with wild-type and vaccine virus are needed before a definitive conclusion can be made.

The current study was designed to determine if a difference exists between the virus shedding patterns of the vaccine virus (PRIME PAC^{TM}, Schering Plough Animal Health) and wild-type virus (PRRSV VR-2332). The study was also conducted to determine when the probability of shedding the virus is lowest and acclimated pigs exposed to wild-type or vaccine virus could be introduced into a herd.

Materials and Methods

Animals

Twenty-four 100-day-old crossbred pigs (principals) and twelve 28-day-old crossbred pigs (sentinels) were obtained from a PRRSV-negative herd. All pigs were similar in genetic background and transported to the isolation facilities on the same day. The sentinels were housed separately and maintained on a raised deck. All 36 pigs were injected intramuscularly with antibiotic (Excenel®, Pharmacia-Upjohn) for 3 consecutive days as

a preventive health measure. The principals were fed ad libitum for the first month and thereafter at a maintenance level.

Experimental designs

The purpose of this study was to compare shedding patterns of MLV-vaccinated versus wild-type virus infected pigs. Principals were randomly assigned in equal numbers (n=8) to three treatment groups, designated groups I, II and III. Group I served as the non-infected controls. Group II was comprised of pigs that were vaccinated intramuscularly once with 2 ml of MLV (PRIME PAC™, Schering-Plough Animal Health). Group III contained pigs that were challenged intramuscularly with 2 ml of PRRSV strain VR-2332 diluted to $10^{5.4}$ $TCID_{50}$/ml. Principals were monitored over a 105-day period for antibody response and virus shedding. Sera were collected weekly beginning at day 0, and assayed for virus, virus RNA and specific antibodies. Oropharygeal scrapings were collected weekly beginning at 49 days p.i., and assayed for virus and virus RNA. Virus shedding was also monitored by placing 2 groups of 2 sentinel pigs each in contact with principals on days 77 and 91. Nose-to-nose contact was possible through a partition. Each group of sentinel pigs remained in contact with the principals for 14 days. Subsequently, they were removed and housed in a separate unit for 2 additional weeks to allow time for seroconversion. Sentinel pigs were tested weekly by ELISA for serological evidence of virus infection. The viremic and serological responses of both groups of virus-infected pigs were also characterized over the experimental period. Two pigs from each group were removed on day 77 to allow space for the sentinel pigs. The remaining pigs were killed on day 109, and tonsils were collected and assayed for virus and virus RNA.

Statistical analysis

Analysis of variance (ANOVA) was performed to determine if there were significant differences among the 3 groups for each day separately. If the p-value for an ANOVA table was less than 0.05, the differences between treatment groups were evaluated by the Tukey-Kramer Honestly Significant Difference (HSD) multiple comparison test. All data analyses were performed using JMP® statistical software (SAS Institute, Inc.).

Sample collection

Oropharyngeal samples were collected weekly, beginning at 49 days p.i. Individual pigs were restrained by snare and samples were collected by scraping the palatine tonsil with an elongated spoon. Samples were mixed with 1ml of sterile DMEM supplemented with 50 μg/ml gentamicin (Gentocin®, Schering-Plough Animal Health) and filtered through a 0.22μm nitrocellulose membrane. Filtrates were then stored at -80°C until used.

Blood was collected in SST tubes (Vacutainer™, Becton Dickinson, Franklin Lakes, NY), allowed to clot at ambient temperature, and then maintained for 1 hour at 4-5°C for clot contraction. Blood tubes were centrifuged at 400 × g for 10 minutes. Sera were separated from the blood within 3 hours of collection and sera were stored in 1 ml aliquots at -80°C until used.

Virus isolation (VI)

Oropharyngeal samples were assayed for the presence of virus by inoculating 200 μl of filtered samples in duplicate onto 48-hours-old confluent MARC-145 cell monolayers contained in 48-well tissue culture plates. The inoculated cells were incubated for 1 hour at 37°C. Subsequently, inoculums were replaced with DMEM supplemented with 3% fetal bovine serum (FBS) and 50 μg/ml gentamicin. The plates were then incubated for 2 days at 37°C. Media were removed and the cell monolayers were fixed with cold acetone-methanol solution (70:30) for 10 minutes and air-dried. Virus was detected in monolayers by indirect fluorescent microscopy using the PRRSV-specific monoclonal antibody (Mab) ISU-15A (Yang et al., 1999). Two aliquots of each sample were assayed. The serum samples were assayed for virus in an identical manner, with the exception that 100 μl of inoculum were used.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Analysis of serum, oropharyngeal scrapings and tonsil tissues for virus RNA was conducted at South Dakota State University using the PCR protocol described by Christopher-Hennings et al. (1995).

Antibody detection

The SN test was performed according to method described by Yoon et al. (1995) but with minor modifications. Serum samples were heat-treated for 30 minutes at 56°C. Sera were diluted twofold in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin. Each dilution of serum was mixed with an equal

volume of PRRSV diluted to contain 10^2 $TCID_{50}$/0.1ml. Serum/virus mixtures were incubated for 60 minutes at 37°C. Two hundred µl of each serum virus mixture were added in duplicate to the wells of a 96-well microtiter plate containing 48-hour-old confluent MARC-145 cell monolayers. The plates were incubated for 2 days at 37°C. The cell monolayers were fixed with cold acetone-methanol solution (70:30) and the presence of virus was detected by indirect fluorescent microscopy as previously described. The titers of sera were expressed as the reciprocal of the highest serum dilution that yields a 100% reduction in the number of observed fluorescent focus. Assays were replicated three times. Geometric mean titers were calculated.

Sera were also assayed by ELISA for PRRSV-specific antibody. A commercial ELISA (HerdCheck®, IDEXX Laboratories, Westbrook, ME) was used as directed by the manufacturer. The presence or absence of antibody was determined by calculating the sample-to-positive (S/P) ratio. Samples were considered to be positive for PRRSV antibody if the S/P ratio was greater than 0.4.

Results

Response of pigs to infection

The response of pigs to infection with vaccine or wild-type virus as measured by the duration of viremia is summarized in Table 1. Virus was first isolated in the serum of 6 of 8 pigs in group II on day 14, and in a 7th pig on day 22. Virus was not isolated in the serum of these 7 pigs after day 22. No virus was isolated from the 8th pig in this group throughout the study. However, virus RNA was detected by PCR in the serum of this pig. Virus was first isolated in all 8 pigs of group III on day 7. Virus was subsequently isolated from 5 of these pigs, sporadically, between days 14 and 28. Virus was not isolated from any pig of group III after day 28. Pigs in group I remained PRRSV-negative throughout the 105-day experimental period (results are not shown).

The response of pigs to infection with vaccine or wild-type virus as measured by the duration of virus RNA present in serum is summarized in Table 1. Virus RNA was first detected by PCR in 3 of 8 pigs of group II on day 7, and in 3 additional pigs on day 14. Virus RNA was also detected by PCR in 1 of these 6 pigs on days 22 and 28. Virus RNA was not detected in any of these 6 pigs after day 28. Virus RNA was not detected in 2 of the 8 pigs after exposure, even though virus was isolated in the serum of these pigs. In contrast, virus RNA was first detected by PCR in the serum of 6 of 8 pigs in group III on day 7, and in 1 additional pig on day 28.

Table 1
Detection of virus and viral RNA in the serum of pigs infected with vaccine (group II) and wild-type virus (group III)

Group	Pig	Days post infection							
		0	7	14	22	28	35	42	49[c]
II	63	-/-	-/-	V/R[a]	-/-	-/-	-/-	-/-	-/-
	64	-/-	-/R	V/R	-/-	-/-	-/-	-/-	-/-
	65	-/-	-/-	V/-	-/-	-/-	-/-	-/-	-/-
	71	-/-	-/-	V/-	-/-	-/-	-/-	-/-	-/-
	82	-/-	-/-	-/R[b]	-/-	-/-	-/-	-/-	-/-
	84	-/-	-/R	V/R	V/-	-/-	-/-	-/-	-/-
	85	-/-	-/R	V/R	-/-	-/-	-/-	-/-	-/-
	88	-/-	-/-	-/R	V/R	-/R	-/-	-/-	-/-
III	70	-/-	V/R	V/R	-/-	V/-	-/-	-/-	-/-
	72	-/-	V/R	-/-	V/R	-/R	-/-	-/-	-/-
	73	-/-	V/-	-/-	-/-	-/-	-/-	-/-	-/-
	75	-/-	V/R	-/-	-/-	-/-	-/-	-/-	-/-
	77	-/-	V/R	-/R	V/-	-/R	-/-	-/-	-/-
	79	-/-	V/R	-/-	-/-	-/-	-/-	-/-	-/-
	81	-/-	V/R	-/-	-/R	V/-	-/-	-/-	-/-
	83	-/-	V/-	V/-	-/-	V/R	-/-	-/-	-/R

[a] V/R = Virus/virus RNA
[b] Pig seroconverted on day 14.
[c] All pigs tested on day 56-105 were negative for virus and virus RNA.

Virus RNA was not detected in 1 pig of this group even though virus was isolated in the serum of this pig on day 7. Virus RNA was not detected in 7 of 8 pigs after day 28, but was detected in 1 pig on day 49.

Serological response of pigs to infection

The serological response of pigs to infection with vaccine or wild-type virus as measured by the ELISA is summarized in Fig. 1. Results are represented as the mean $\pm$ one standard error of mean (S.E.M.). Antibodies were first detected in 3 pigs of group II on day 14, in 3 additional pigs on day 21, and in 1 pig on day 35. One of the 8 pigs (# 64) in this group did not seroconvert even though virus was isolated in the serum of this pig on day 14. The mean ELISA titer reached a maximum value of 0.909 ± 0.13 on day 28 and then declined to a mean value of 0.37 ± 0.08 by day 105. Antibodies were first detected on day 14 in all pigs of group III. The mean ELISA titer of group III reached a maximum value of 1.88 ± 0.086 on day 28 and then declined to a mean value of 0.81 ± 0.09 by day 105. The mean ELISA titer of group III between day 14 and 105 was significantly higher ($p\leq0.05$) than that of group II.

The response of pigs to infection with vaccine or wild-type virus as measured by the SN antibody response is summarized in Fig. 2. Results are represented as the geometric mean $\pm$ one S.E.M. The SN antibodies were first detected on day 22 in all 8 pigs of group II. The geometric mean SN antibody titer was 1.68 ± 0.29 on day 22, and increased to maximum level of 3.93 ± 0.17 on day 63. The geometric mean SN antibody titer was 3.6 ± 0.24 on day 105. The SN antibodies were first detected on day 22 in 3 of 8 pigs of group III, and in all 8 pigs by day 35. The geometric mean SN antibody titer was 0.8 ± 0.29 on day 22, and increased to maximum level of 3.87 ± 0.3 by day 63. The geometric mean SN antibody titer was 3.66 ± 0.33 on day 105.

Virus shedding

All sentinel pigs that were placed in contact with pigs of groups I, II and III on days 77 and 91 did not seroconvert over a 28-day period of observation (Tables 2 and 3). Virus and virus RNA were not detected in the tonsils of any sentinel pigs at necropsy (results are not shown). Virus was not isolated from oropharyngeal scrapings of any pig infected with vaccine or wild-type virus. The shedding patterns of pigs infected with vaccine virus and pigs infected with wild-type virus, as reflected by the presence of virus RNA in oropharyngeal scrapings, are summarized in Table 4. Virus RNA was detected in 3 of 8 pigs of group II. Virus RNA was detected in 1 of these pigs on days 49 and 56, and in 2 of these pigs on day 70. Virus RNA was not

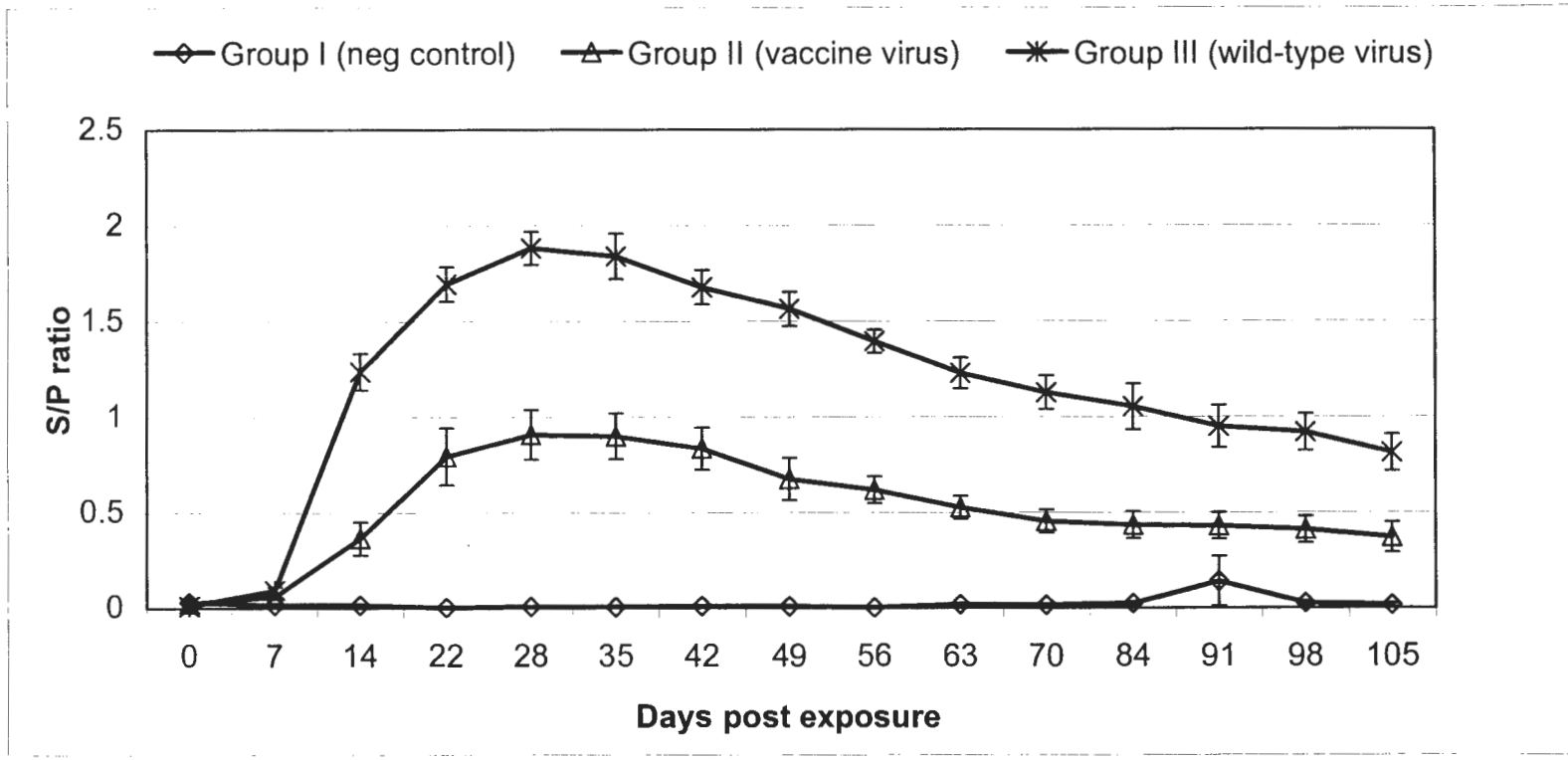

Fig. 1. Antibody response as measured by ELISA. The titer is reported as an S/P ratio. An S/P ratio of ≥0.4 indicates a positive result. The number of pigs in each treatment was 8 on day 0. The number of pigs in each treatment was 6 after day 77. Results are expressed as the mean titer ± one S.E.M.

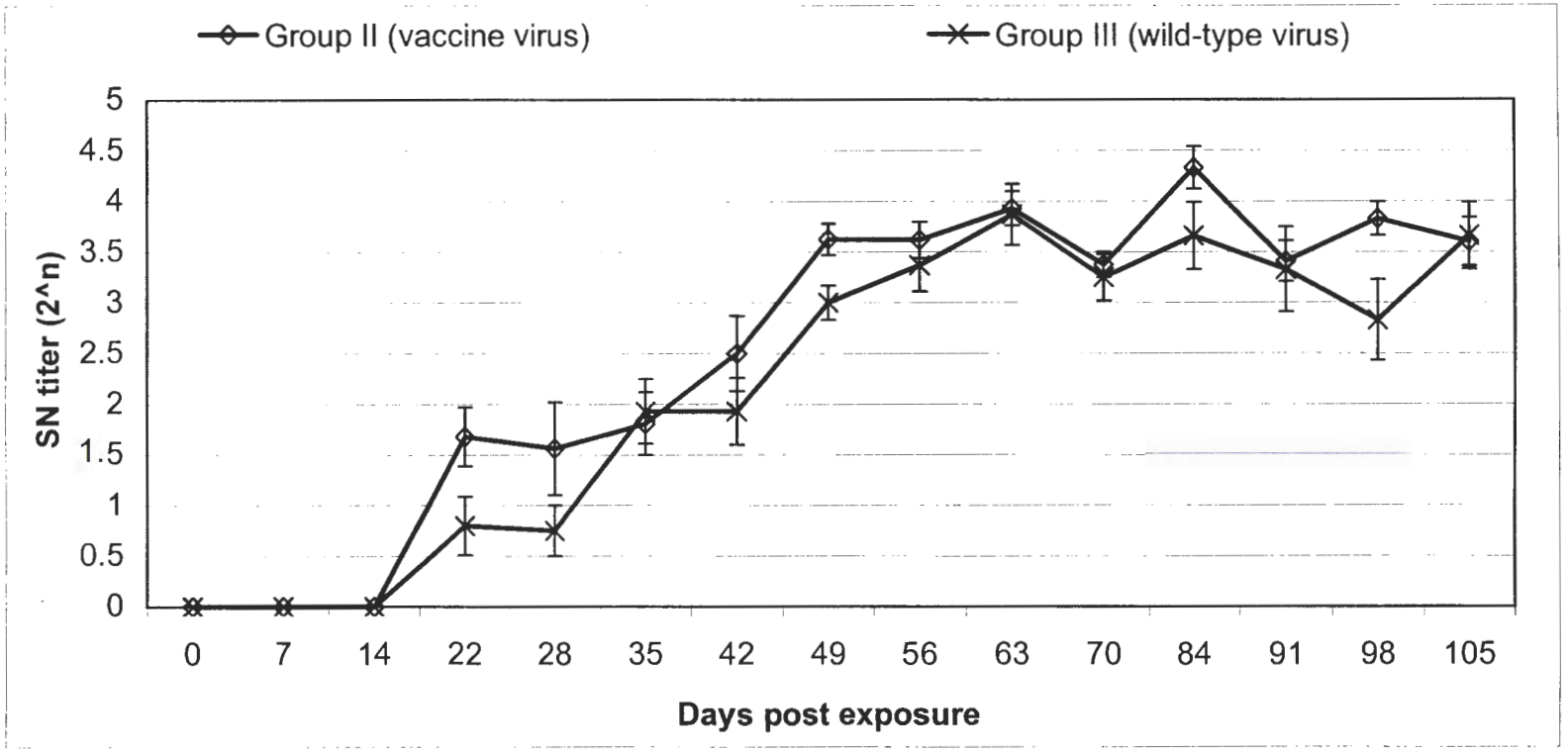

Fig. 2. Antibody response as measured by the serum neutralizing (SN) test. The number of pigs in each treatment was 8 on day 0. The number of pigs in each treatment was 6 after day 77. Results are expressed as the mean titer $\pm$ one S.E.M.

Table 2
Antibody response of sentinel pigs[a] as measured by ELISA[b]

Group	Sentinel pig no.	0[c]	7	14	21	28
I	57-73	0.017	0.053	0.042	0.044	0.041
	57-79	0.011	0.013	0.018	0.024	0.26
II	57-74	0.036	0.042	0.044	0.02	0.016
	57-80	0.024	0.036	0.032	0.044	0.022
III	57-69	0.176	0.04	0.124	0.12	0.09

[a] Sentinel pigs were placed in contact with non-infected control pigs (group I), pigs infected with vaccine (group II) or wild-type virus (group III) on day 77. Sentinel pigs were housed with principal pigs for 2 weeks and thereafter in a separate unit for 2 additional weeks.

[b] The titer is reported as an S/P ratio. An S/P ratio of ≥0.4 indicates a positive result.

[c] Days post exposure to principal pigs.

Table 3
Antibody response of sentinel pigs[a] as measured by ELISA[b]

Group	Sentinel pig no.	0[c]	7	14	21	28
I	57-70	0	0	0.056	0.041	0.037
	57-71	0	0	0	0.006	0
II	57-75	0.004	0	0	0.026	0.02
	57-76	0.013	0	0	0	0
III	57-72	0	0	0	0.053	0.048
	57-77	0	0	0.018	0	0.009

[a] Sentinel pigs were placed in contact with non-infected control pigs (group I), pigs infected with vaccine (group II) or wild-type virus (group III) on day 77. Sentinel pigs were housed with principal pigs for 2 weeks and thereafter in a separate unit for 2 additional weeks.

[b] The titer is reported as an S/P ratio. An S/P ratio of ≥0.4 indicates a positive result.

[c] Days post exposure to principal pigs.

Table 4
Detection of virus RNA in oropharyngeal scrapings and tonsil tissues of pigs infected with vaccine (group II) and wild-type virus (group III)

Group	Pigs	Days post infection									
		49	56	63	70	77T[a]	84	91	98	105	109 T[a]
II	63	-	-	-	-	NT[c]	-	-	-	-	-
	64	R[b]	R	-	-	NT	-	-	-	-	R
	65	-	-	-	-	NT	-	-	-	-	-
	71	-	-	-	R	NT	-	-	-	-	R
	82	-	-	-	R	NT	-	-	-	-	-
	84	-	-	-	-	-					
	85	-	-	-	-	-					
	88	-	-	-	-	NT	-	-	-	-	R
III	70	-	R	-	-	NT	-	-	-	-	-
	72	-	-	R	R	NT	-	-	-	-	R
	73	-	R	-	-	R					
	75	-	-	-	-	NT	-	-	-	-	-
	77	-	-	-	-	R					
	79	-	-	-	R	NT	-	R	R	R	-
	81	-	-	-	-	NT	-	-	-	-	-
	83	-	R	R	R	NT	R	-	-	R	R

[a] Pigs were killed and tonsils were assayed for virus and virus RNA. No virus was detected in any pig.
[b] R= virus RNA
[c] NT= not tested.

detected in any pig of group II after day 70. In contrast, virus RNA was detected in 5 of 8 pigs of group III. Virus RNA was detected in 1 pig on each sampling day between days 56 and 70. Virus RNA was also detected in 2 different pigs on day 56, in 1 pig on day 63 and in 2 different pigs on day 70. Virus RNA was also detected in 2 of the 5 virus RNA-positive pigs between days 70 and 105. Virus RNA was detected in one of these pigs on days 91, 98 and 105, and in a second pig on days 84 and 105.

Two pigs from each group were killed on day 77, and the remainings were killed on day 109. Virus was not isolated in the tonsils of any pig killed on days 77 and 109. Virus RNA was not detected in the tonsils tissue of the 2 pigs of groups II on day 77, but it was detected in the tonsils of 3 of the 6 remaining pigs of group II on day 109. Virus RNA had been detected earlier in oropharyngeal scrapings from 2 of these 3 pigs. In contrast, virus RNA was detected in the tonsils of 2 pigs of group III on day 77, and in 2 of the 6 remaining pigs of group III on day 109. Virus RNA had also been detected earlier in the oropharyngeal scrapings of all 4 of these pigs.

Discussion

Control of PRRSV infection is based on the early exposure of replacement pigs to infectious virus at an early age, with the objective to infect and immunize the replacement pigs prior to their introduction into the breeding herd. Currently, infection is done by using modified live PRRSV vaccine (MLV) or by relying on natural exposure methods after commingling the replacement animals with previously infected pigs. However, natural exposure is not always successful since it is difficult to implement, and the detection of infected pigs under field conditions is labor intensive. Therefore, PRRSV MLVs constitute an attractive tool that, if proven to induce protective immunity and a shorter period of virus shedding, could be used as an alternative to infect and immunize gilts prior to their introduction into the breeding herd. This study was designed to compare the virus-shedding patterns of pigs infected with wild-type (PRRSV VR-2332) and vaccine virus (PRIME PAC™ , Schering-Plough Animal Health). A shorter shedding time would not only result in safer gilt introduction but also in the reduction of the gilt acclimatization period.

There was a difference between the virus-shedding patterns of wild-type and vaccine viruses. Virus RNA was not detected in oropharyngeal scrapings of pigs infected with vaccine virus after day 70, but it was detected in 2 of the 6 remaining pigs that were infected with wild-type virus up to 105 days following exposure

(Table 2). Even though virus RNA was not detected in any pig infected with vaccine virus between day 70 and 105 post following exposure, the fact that virus RNA was detected in the tonsils of pigs exposed to either wild-type or vaccine virus at necropsy suggests that virus was still present in both groups of pigs but was not transmitted to contact sentinel pigs. Whether virus shedding can be reinitiated needs to be further evaluated.

It is interesting to note that the difference between the duration of vaccine virus shedding to sentinel pigs described by Srinivasappa et al. (1999) and the duration of wild-type virus shedding to sentinel pigs reported by other investigators (Albina et al., 1994; Lager and Mengeling, 1996; Terpstra et al., 1992; Wills et al., 2000) ranged from 20 to 30 days. This difference in the shedding pattern of wild-type and vaccine viruses is similar to the difference in the shedding pattern of wild-type and vaccine viruses reported in the present study as determined by the detection of virus RNA in oropharyngeal scrapings.

In our study, neither wild-type nor vaccine virus was isolated in the oropharyngeal scrapings of any pig beyond 49 days after exposure. Only virus RNA was detected by PCR in oropharyngeal scrapings. These observations were unexpected. The duration of wild-type virus shedding described by other investigators is relatively longer, as compared to the results of the present experiment. For example, Wills et al. (1997b, 1997a) used the PRRSV strain ISU-P to challenge pigs and detected virus shedding in oropharyngeal scrapings. These investigators observed that virus was isolated from oropharyngeal scrapings of 2 of 4 pigs up to day 84 (Wills et al., 1997a) and from 1 of 4 pigs up to day 157 (Wills et al., 1997b). The discrepancy between the present study and the studies by Wills et al. (1997a, 1997b) could possibly be explained by differences among PRRSV isolates, the host, or methodology in sampling. Differences in sensitivity of virus isolation protocols might be another reason, because pulmonary alveolar macrophages (PAMs) were used to isolate viruses in the studies by Wills et al. (1997b). In contrast, MARC 145 cells were used in the current study. Pulmonary alveolar macrophages are relatively more sensitive for virus isolation than MARC 145 (Botner, 1997). Another explanation might be that PCR positive and VI negative for PRRSV samples indicate that the level of the replicating virus is below the sensitivity of the VI assay. The PCR test is more sensitive than virus isolation (Spagnuolo-Weaver et al., 1998).

Some samples were negative PCR and positive VI (Table 1). These results were unexpected since PCR is more sensitive than VI (Spagnuolo-Weaver et al., 1998). An explanation might be that PCR or VI was not working properly or there were errors in the technique.

The PCR results from tonsil samples collected at post mortem indicate that both pigs infected with either vaccine virus or wild-type virus still carried virus RNA on 109 days post exposure. The detection of virus RNA is not necessarily equated to the isolation of infectious virus. The existence of viral genomic material needs to be assessed further to determine if the pigs were infectious and contagious. Using a swine bioassay, Horter et al. (2000) demonstrated that homogenates from tonsils collected from pigs infected with PRRSV strains VR-2332 at 105 days post exposure remained infectious. The investigators also found that all (100%) of the pigs carried virus on day 63. The carrier status of the infected pigs decreased over time, and approximately 90% of the pigs carried the virus at 105 days post exposure. However, it was not determined if the RNA-positive samples represented infectious virus.

Sentinel pigs housed with principal pigs on days 77 and 91 remained uninfected as indicated by their failure to seroconvert. Although wild-type virus was not transmitted to sentinel pigs, the detection of RNA positive samples in the oropharyngeal cavity might represent a risk for naïve animals housed next to previously-infected animals. Transmission in this study may not have been fully maximized since the sentinel pigs were placed in separate pens with an open gate and allowed only nose-to-nose contact. Housing principals and sentinels in the same pen would have been a better way to evaluate transmission.

Srinivasappa et al. (1999) reported that vaccine virus was shed to sentinel pigs for up to 39 days. Previous studies by several investigators involving several wild-type strains of PRRSV on the duration of PRRSV shedding to sentinel pigs suggest that the average virus shedding to sentinel pigs ranges from 60 to 70 days following exposure (Albina et al., 1994; Lager and Mengeling, 1996; Terpstra et al., 1992; Wills et al., 2000; Yoon et al., 1993;). A study by Zimmerman et al. (1992) using PRRSV strain ISU-P as the inoculation strain concluded that virus transmission can occur from a sow to finisher pigs, even if a sow was infected 99 days previously.

Factors such as strain differences, age of pigs, and genetic background might affect variability in the duration of virus shedding. Strain differences exist among PRRS viruses. The capability of a virus to establish

persistent infection in a host may also vary depending on the virus strain or host-intrinsic factors. Some strains may persistently infect pigs for a longer duration than others.

The shedding of PRRSV appears to be associated with age. Younger pigs seem to remain viremic for a longer period of time and are more susceptible to PRRSV than older pigs (Lager and Mengeling, 1996). The viremic phase appears to be shorter in older pigs, which might be due to improved immunity. Pigs infected *in utero* were found to shed the virus to in-contact penmates up to 112 days post partum (Benfield et al., 1997). In our study, 100 day-old pigs were used. These pigs were older than pigs used in studies by Horter et al. (2000), and Wills et al. (1997a, 1997b), in which the pigs were 3 to 4 week of age. Therefore, the difference in duration of virus shedding might be due to the age of pigs.

Two studies demonstrated the effect of genetics on differences in susceptibility to PRRSV. Halbur et al. (1998) challenged purebred Duroc, Hampshire, and Meishan pigs with a highly virulent strain of PRRSV. None of these pigs were resistant to PRRSV infection; however, differences in severity of lung lesions, number of virus-infected cells in lungs, and incidence of myocarditis and encephalitis were noticeable. Hampshire pigs had more severe lung lesion scores than the Duroc and Meishan pigs. Meishan pigs had fewer numbers of virus-infected cells in lungs, but had a higher incidence of myocarditis and encephalitis. A study by Christopher-Hennings et al. (2001) concluded that Yorkshire boars were more resistant to PRRSV shedding in semen than Landrace and Hampshire boars. Crossbred pigs were used in the present experiment. Studies by Horter et al. (2000), Wills et al. (1997a and 1997b) also used crossbred pigs. The genetic differences of the pigs among those studies were not determined in this study.

It is interesting to note that the mean ELISA titers of pigs exposed to vaccine virus (PRIME PAC™) were significantly lower than that of pigs exposed to wild-type virus. However, there was no difference in SN antibody response. These observations were unexpected. On the other hand, Wasmoen et al. (1998) conducted an experiment to determine antibody response of pigs vaccinated with PRIME PAC™ or RespPRRS® vaccine, and found that PRIME PAC™ vaccine induced lower antibody levels than RespPRRS® vaccine.

The presence of virus RNA in the tonsils of pigs exposed to either wild-type or vaccine virus at necropsy on day 109 suggests that virus remained in the pigs and, therefore, shedding could potentially be reinitiated at any time. However, this could not be confirmed by contact exposure of negative sentinel pigs.

Additonal studies are needed to determine if virus RNA detected in tonsils at necropsy remains infectious, as well as to clarify if factors such as age, strain of virus, and degree of contact affect virus shedding.

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CHAPTER 4. THE EFFECT OF KILLED VACCINE TREATMENT ON VIRUS SHEDDING IN PIGS PREVIOUSLY INFECTED WITH VIRULENT PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

A paper to be submitted to Veterinary Microbiology

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Abstract

The objective of this study was to determine if virus shedding in pigs previously infected with virulent porcine reproductive and respiratory syndrome virus (PRRSV) could be reduced by enhancing immune response through the use of a commercially available killed vaccine (KV). Two experiments were conducted. In the first experiment, 2 groups of 5 non-infected control pigs and 2 groups of 20 infected pigs each were used. One group of infected pigs was treated with KV on days 14 and 28 post infection (p.i.). Viremias of both KV-treated infected and non-treated infected groups were compared to evaluate the effect of the KV treatment. Sera were collected weekly and assayed for the presence of serum neutralizing (SN) antibody during a period of 42-days. The second experiment was conducted in an identical manner to the first experiment with the following exceptions. The number of pigs per group was reduced to 8. Two vaccine treatment groups were used. One group was treated with the KV on days 7 and 21 p.i., and the other group was treated with KV on days 14 and 28 p.i. Oropharyngeal scrapings were collected and evaluated for virus and virus RNA on days 42 and 56. The number of interferon gamma (IFN-γ) producing cells was monitored biweekly, starting on day 42 p.i. There was no difference in the magnitude and duration of viremia between the KV-treated infected group and non-treated infected group in the first experiment, nor was a difference found in the second experiment. No virus was detected in oropharyngeal scrapings of any pig in any group, nor was there a significant difference in the detection of virus RNA. Virus RNA was detected in 2 of 6 KV-treated infected pigs, and in 2 of 6 non-treated infected pigs. These observations indicate that the KV treatment had no effect on virus shedding. In both experiments, increases were observed in SN titer over time and in the numbers of IFN-γ producing cells. The mean SN antibody titer of KV-treated infected groups was significantly greater ($p \leq 0.05$) than that of the non-treated infected group on day 42 p.i. in the first experiment, and on days 42-56 p.i. in the second experiment. There was no difference from days 0 to 35 in the first experiment, and from days 0 to 28 and days 63 to 78 in the second experiment. The mean number of IFN-γ producing cells was apparently higher in the KV-treated

infected groups than in the non-treated infected group on days 42 and 63. These observations indicate that the KV treatment had no effect on virus shedding, as determined by the presence of virus RNA in the oropharyngeal scrapings. However, infected pigs responded immunologically to the KV treatment, as indicated by the increase in the mean SN antibody titer and the apparent increase in IFN-γ producing cells.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the *Arteriviridae* (Cavanagh, 1997). Since its first emergence in late 1980s, PRRSV has had a significant economic impact on the swine industry worldwide. Clinical signs of PRRSV infection include reproductive failure in dams, respiratory disease in grower and finisher pigs, and increased pre-weaning mortality (Loula, 1991). An identifying characteristic of PRRSV is the persistent nature of virus infection (Wills et al., 1997b) and delay in the development of serum neutralizing (SN) antibody (Nelson et al., 1994; Osorio, 1999; Yoon et al., 1995). These characteristics of the virus make it difficult to control the disease that it causes.

Several management protocols have been used to control and eliminate PRRSV. These protocols include total depopulation followed by repopulation with PRRSV-free replacement pigs (Andreasen et al., 1998), partial herd depopulation such as the depopulation of entire nursery units to prevent the introduction of virus into finisher units (Hassing et al., 2000), test and removal of infected pigs (Dee et al., 2001), and total herd vaccination (Philips et al., 2000). Temporary herd closure has also been used with some success (Torremorell and Baker, 2000; Torremorell et al., 2000). This management protocol consists of closing the herd to any replacement for a specified period of time to establish herd immunity and decrease virus shedding. Subsequent replacement pigs must be acclimatized, during which time they are intentionally exposed to the farm-specific strain of the virus before introduction into the herd. The length of the acclimatization period is dependent on the period of virus shedding and the development of immunity.

Several studies have reported that infected pigs shed PRRSV for variable periods of time following infection. Wills et al. (1997a, 1997b) conducted studies in which pigs were infected with the PRRSV strain VR-2402. Virus shedding was monitored over a 124-day period in the first study, and over a 213-day period in the second study. In both studies, virus shedding was monitored by assaying oropharyngeal scrapings for virus and virus RNA, starting at day 55 post infection (p.i.). In the first study, virus was recovered from 2 of 4 pigs on

days 55 and 84 (Wills et al., 1997a) but no virus was isolated after day 84. In the second study, virus was isolated periodically from 2 of 4 pigs during days 56 to 84, and from a third pig during days 56 to 157 (Wills et al., 1997b). However, no virus was recovered after day 157. The combined results of these studies indicate that virus could be shed in as many as 70% of infected pigs for at least 84 days, but virus shedding dramatically decreases thereafter.

Attempts to reduce virus shedding by vaccine treatment have been made by Swenson et al. (1995) and also Nielsen et al. (1997). Swenson et al. (1995) compared virus shedding in semen of KV-treated pigs with non-treated pigs between days 0-32 following infection with wild-type virus. They found infectious virus in semen of 2 of 4 KV-treated pigs on days 4 and 7, respectively. Infectious virus was detected periodically in 2 other pigs through days 25 and 28, respectively. In contrast, infectious virus was detected continuously in semen of all 3 non-treated pigs through day 32. The authors suggested that treatment with KV appeared to have reduced virus shedding, although the difference in the number of days of shedding was not statistically significant. The results by Swenson et al. (1995) were not duplicated by Nielsen et al. (1997), who conducted a similar experiment using 5 pigs per each group to evaluate the effect of modified-live vaccine (MLV) and killed vaccine (KV) on virus shedding in semen. These investigators found that KV treatment had no effect on the duration of viremia and virus shedding. Infectious virus was detected continuously in 2 of 5 KV-treated pigs through day 10 p.i., one pig though day 14 p.i., and in the 2 remaining pigs through day 21 p.i. Infectious virus was detected in 1 non-treated control pig through day 10, and in the 4 remaining pigs through day 14. However, a reduction in viremia and virus shedding was observed in pigs that were treated with MLV. Infectious virus was detected in only 2 of 5 MLV-treated pigs on days 7 and 14, respectively.

Protocols to increase SN antibody titer are important because SN antibody response appears to be correlated well with resistance to infection. Recently, Osorio et al. (2001) conducted an experiment in which 6 pregnant sows were injected with polyclonal-neutralizing antibodies and subsequently challenged with PRRSV. All 6 sows receiving PRRSVspecific SN antibodies were fully protected from reproductive failure. Furthermore, no infectious virus was detected in the serum of the sows following challenge. In contrast, sows receiving anti-pseudorabies antibodies had marked reproductive failure. Studies by Joo et al. (1999) and Direksin et al. (2000) also reported similar results. In their study, 20 sows were recruited from an endemically

infected herd and challenged with PRRSV. Sows with SN antibody titers ranging from 2-64 were not viremic following PRRSV re-exposure. In contrast, sows without SN antibody titers were viremic following rechallenge. These observations suggest that SN antibodies are correlated well with reduced viremia and might have an effect on virus shedding.

The objectives of the current study were to determine if virus shedding from pigs previously infected with virulent PRRSV could be reduced by the enhancement of immune response through the use of KV.

Materials and Methods

Experimental designs

Two experiments were conducted using fifty 10-week-old pigs in the first experiment and thirty-two 3-4-week-old pigs in the second experiment. The pigs in both experiments were crossbred pigs from the same PRRSV-free herd that had the same genetic background.

Experiment 1

Fifty 10-week-old crossbred pigs were randomly assigned to 4 treatment groups, designated as groups I, II, III, and IV. Group I (n=5) served as a non-infected control. Group II (n=5) served as a KV control. Group III consisted of pigs (n=20) that were challenged intranasally on day 0 with 1 ml of PRRSV strain VR-2385 (kindly provided by Drs. P.G. Halbur and P. S. Paul, Iowa State University) containing $10^{5.4}$ $TCID_{50}$ total and vaccinated intramuscularly with 2 consecutive doses of KV on days 14 and 28 (PRRomiSe®, Intervet Corporation). Group IV was comprised of pigs that were challenged with PRRSV in an identical manner, but they were not vaccinated with KV. Pigs were monitored from 0 to 42 days p.i. by collecting serum at weekly intervals and assaying for virus and antibody. The pigs were killed on day 44, and their tonsils were collected and assayed for virus and virus RNA.

Experiment 2

Thirty-two 3-4- week-old crossbred pigs were randomly assigned to 5 treatment groups designated as groups I, II, III, IV, and V. Group I (n=4) served as the non-infected control and Group II (n=4) served as the KV control. Group III was comprised of pigs (n=8) that were challenged intranasally on day 0 with 1 ml of PRRSV strain VR-2385 containing $10^{5.4}$ $TCID_{50}$ total and vaccinated intramuscularly with 2 consecutive doses of KV on days 7 and 21 (PRRomiSe®, Bayer Corporation, Shawnee, KS). Group IV was comprised of pigs

(n=8) that were challenged with PRRSV in an identical manner and vaccinated with KV on days 14 and 28. The pigs in Group V (n=8) were challenged with PRRSV in an identical manner, but were not vaccinated. All pigs were monitored from 0 to 63 days p.i. by collecting serum at weekly intervals and assaying for virus, virus RNA and antibody. Oropharyngeal scrapings were collected on days 42 and 56, and assayed for virus and virus RNA. In addition, from day 42 onward, pigs were monitored biweekly for the number of IFN-γ producing cells.

All pigs in the second experiment were challenged with PRRSV isolate 16244B (kindly provided by Dr. F. A. Osorio, University of Nebraska-Lincoln) on day 65. Pigs were monitored for 2 additional weeks, and their sera were collected and assayed for virus, virus RNA and antibody. Pigs were killed on day 78, and their tonsils and lung lavage samples were collected and assayed for virus and virus RNA.

Statistical analysis

Analysis of variance (ANOVA) was performed to determine if there were significant differences among the 5 groups for each day separately. If the p-value for an ANOVA table was less than or equal 0.05, the differences between treatment groups were evaluated using the Tukey-Kramer Honestly Significant Difference (HSD) multiple comparison test. All data analyses were performed using JMP® statistical software (SAS Institute, Cary, NC).

Sample collection

Individual pig was restrained by snare. Oropharyngeal scraping samples were collected by scraping the palatine tonsil with an elongated spoon. Samples were mixed with 1ml of sterile DMEM supplemented with 50 μg/ml gentamicin (Gentocin®, Schering-Plough Animal Health) and filtered through a 0.22μm nitrocellulose membrane and stored at -80°C until assayed.

Blood was collected in serum separation tubes (SST Vacutainer™, Becton Dickinson, Franklin Lakes, NY) and sterile heparinized tubes (BD Vacutainer™ Sodium Heparin, Becton Dickinson, Franklin Lakes, NY). The blood in the SST tubes was allowed to clot at ambient temperature, after which it was placed in a refrigerator for 1 hour at 4-5°C for clot contraction. Blood tubes were centrifuged for 10 minutes at 400 × g. Sera were separated within 3 hours after collection and stored in 1 ml aliquots at -80°C until used. Sera were assayed for virus and for virus RNA.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood. In brief, whole blood collected in sterile heparinized tubes was diluted 1:2 in sterile phosphate buffer saline (PBS), layered over a 1.086±0.0002 g/ml density gradient (Accu-paque™ Lymphocytes, Accurate Chemical & Scientific Corporation, NY), and centrifuged for 30 minutes at 400 × g. The opaque interface containing mononuclear cells was collected and subsequently washed one time with PBS. The mixture was centrifuged again for 10 minutes at 400 × g and the supernatant discarded. The PBMCs were resuspended in 1 ml RPMI-1640 media supplemented with a solution containing 10% FBS, 2 mM L-Glutamine, 25 mM Hepes and 50 μg/ml gentamicin. Peripheral blood mononuclear cells were assayed for PRRSV specific interferon gamma (IFN-γ) producing cells by the ELISpot technique (Zuckermann et al., 1998).

Virus isolation

Serum samples were assayed for the presence of virus by inoculation in duplicate of 100 μl of filtered samples onto 48-hour-old confluent MARC-145 cell monolayers contained in 48-well tissue culture plates. The inoculated cells were incubated for 1 hour at 37°C. Subsequently, the inoculums were replaced with DMEM supplemented with 3% fetal bovine serum (FBS) and 50 μg/ml gentamicin. The plates were then incubated for 2 days at 37°C. Media were removed, and the cell monolayers were fixed with a cold acetone-methanol solution (70:30) for 10 minutes and then air-dried. Virus was detected in monolayers by indirect fluorescent microscopy using the PRRSV-specific monoclonal antibody (Mab) ISU-15A (Yang et al., 1999, 2000). Two aliquots of each oropharyngeal sample were assayed for virus in an identical manner, with the exception that 200 μl of filtered inoculum were used.

Virus assay

Sera were serially diluted tenfold in DMEM supplemented with 3% fetal bovine serum (FBS) and 50 μg/ml gentamicin. One hundred μl of each serum dilution were inoculated into 5 separate wells containing 48-hour-old confluent MARC-145 cell monolayers in 96-well tissue culture plates. The plates were then incubated for 2 days at 37°C. After the media were removed, the cell monolayers were fixed with a cold acetone-methanol solution (70:30) for 10 minutes and the air-dried. Virus was detected in each monolayer by indirect fluorescent microscopy using the PRRSV specific Mab ISU-15A (Yang et al., 1999, 2000). The titer was expressed as $TCID_{50}$/ml as determined by the method described by Reed and Muench (1938).

Reverse transcriptase –polymerase chain reaction (RT-PCR)

Analysis of serum, oropharyngeal and tonsilar samples for virus RNA was carried out at South Dakota State University using the PCR protocol described by Christopher-Hennings et al. (1995).

Antibody detection

The SN test was performed according to the method described by Yoon et al. (1995), with minor modifications. Serum samples were heat-treated at 56°C for 30 minutes and diluted twofold in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin. Each dilution of serum was mixed with an equal volume of PRRSV diluted to contain 10^2 $TCID_{50}$/0.1ml. Serum/virus mixtures were incubated for 60 minutes at 37°C. Two hundred μl of each serum virus mixture were added in duplicate to the wells of a 96-well microtiter plate containing 48-hour-old confluent MARC-145 cell monolayers and incubated for 2 days at 37°C. The cell monolayers were fixed with a cold acetone-methanol solution (70:30) and the presence of virus was detected by indirect fluorescent microscopy as previously described. The titers of sera were expressed as the reciprocal of the highest serum dilution that yielded a 100% reduction in the number of fluorescent focus. The assays were replicated three times. Geometric mean titers $\pm$ one S.E.M.were calculated.

Sera were also assayed for PRRSV-specific antibody by using a commercial ELISA (HerdCheck®, IDEXX Laboratories, Westbrook, ME) according to the manufacturer's instructions. The presence or absence of antibody was determined by calculating the sample-to-positive (S/P) ratio. A sample was considered to be positive for PRRSV antibody if the S/P ratio was greater than 0.4.

Enzyme-linked immunospot assay (ELISpot) for detecting Interferon-gamma (IFN-γ) producing cells

Porcine reproductive and respiratory syndrome virus specific IFN-γ producing cells in PBMCs were assayed to evaluate cell-medicated immunity. The method used was described by Zuckermann et al. (1998). In brief, 96-well microtiter plates (Immulon 2®, Dynatech Laboratories, Billingshurt, UK) were coated (50μl per well) with a 1:1000 dilution of purified specific anti-porcine IFN-γ Mab P2G10 (kindly provided by Dr. F. A. Zuckermann, University of Illinois) in a 0.05M carbonate-bicarbonate buffer (pH 9.6) for 20 hours at 4°C. Monoclonal antibody was removed and plates were washed twice with sterile phosphate buffer solution (PBS) and then once by RPMI-1640. The plates were blocked with 50μl of RPMI-1640 media containing 5% FBS for 2 hours at 37°C. The blocking solution was removed, and 50μl of PBMC was added to the wells. Each well was

stimulated with 50µl of either RPMI-1640 media, PRRSV VR-2385 at 10^4 $TCID_{50}$/ ml, PRRSV ISU-P at 10^4 $TCID_{50}$/ ml or phytohemagglutinin at 10 µg/ml (PHA-P, Sigma, St. Louis, MO) in duplicate. The plates were incubated for 20 hours at 37°C. Then the plates were washed 4 times with sterile PBS supplemented with 0.05% v/v Tween20 (PBST) and were incubated for 1 hour at 37°C with 50 µl of a 1:8000 dilution of purified specific anti-porcine IFN-γ Mabs P2C11 conjugated with biotin in PBST (kindly provided by Dr. F. A. Zuckermann, University of Illinois). The plates were washed 4 times with PBST and incubated again for 1 hour at 37°C with 50 µl/well of a 1:4000 dilution of streptavidin-horseradish peroxidase (Zymed, San Francisco, CA) in PBST. Finally, the plates were washed 4 times with PBST and incubated for 10 minutes at 37°C with 50 µl/well of 3,3′,5,5′-tetramethylbenzidine membrane peroxidase (BioFX Laboratories, Randallstown, MD). Spots representing individual IFN-γ producing T-cells were counted using a dissecting microscope and expressed as the mean number of IFN-γ producing T-cells per 5×10^5 lymphocytes.

Results

Experiment 1

Response of pigs to PRRSV infection

Seven pigs were removed from the trial due to lameness: 1 pig each on days 28 and 35 from group III; 2 pigs on day 14 and 1 pig each on days 21, 28 and 40 from group IV. No further investigation was done on these 7 pigs.

The response of the pigs to infection as measured by virus titer in serum is shown in Fig. 1. Virus was first isolated on day 8 in the serum of all 20 pigs that were challenged with PRRSV on day 0 and vaccinated with KV on days 7 and 21 p.i. (group III). Virus was isolated in the serum of the same 10 pigs on day 14, and in 4 different pigs on day 21. Virus was not isolated in any pig in group III after day 21. Virus was first isolated in 19 pigs that were challenged with PRRSV on day 0 only (group IV) on day 8, and in 1 remaining pig on day 14. Virus was detected in 8 of these pigs on day 14 and again in 1 pig on days 21 and 28. Virus was not isolated in any pig in group IV after day 28. The mean virus titer of pigs in group III was not different from those of pigs in group IV during a 42-day period. On day 8, the mean virus titer reached the highest level of $10^{4.32}$ $TCID_{50}$/ml

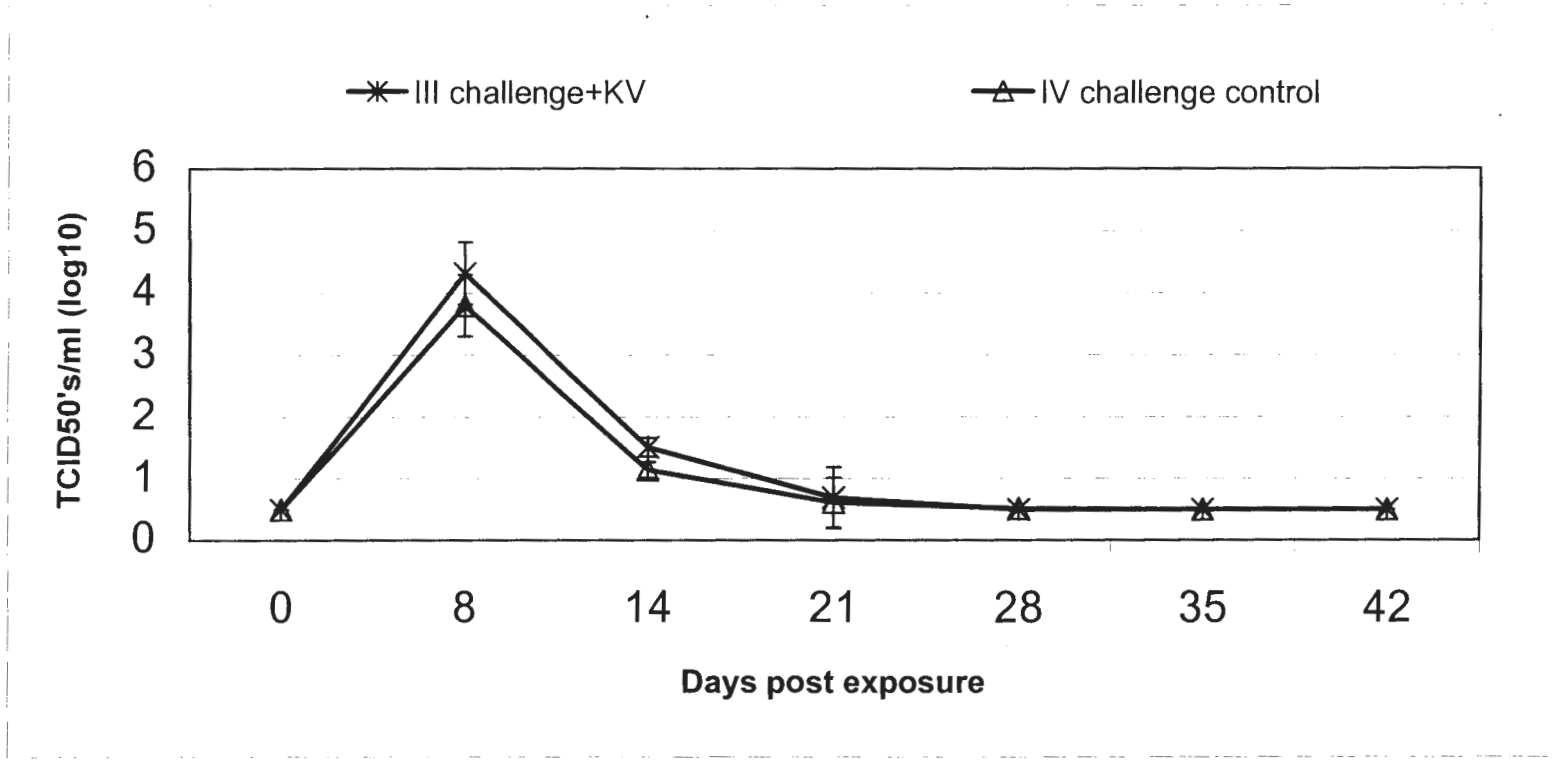

Fig. 1. Virus titer in serum of pigs in experiment 1, expressed as $TCID_{50's}$/ml (log_{10}). Results are represented as the geometric mean titer $\pm$ one S.E.M. Pigs were challenged with PRRSV VR-2385 on day 0. Total number of pigs on day 0 equals 20 in Groups III, and IV. One pig in group III was removed on day 28 and and again on day 35. Two pigs in Group IV were removed on day 14 and one pig was removed on each of days 21, 28 and 40.

in group III, and $10^{3.8}$ $TCID_{50}$/ml in group IV. The mean virus titer in each group decreased thereafter.

Infectious virus was detected in tonsil tissues at necropsy on day 44 p.i. in 8 of 18 pigs in group III, and in 6 of 15 pigs in group IV. Virus RNA was detected by PCR in all tonsil tissues of all pigs in groups III and IV.

ELISA

The serological response of pigs as measured by ELISA is summarized in Fig. 2. Results are represented as the mean ± one standard error of mean (S.E.M.) Antibodies were first detected in 8 pigs in group III on day 8, and in the 12 remaining pigs on day 14. The mean titer of pigs in group III reached the highest level of 1.36±0.05 on day 35 and remained at or near this level through day 42. Antibodies were first detected in 7 pigs in group IV on day 8, and in the 11 remaining pigs on day 14. The mean titer of pigs in group IV reached the highest level of 1.34±0.044 on day 28 and remained at or near this level through day 42. The mean titers of pigs in groups III and IV were similar throughout a 42-day period. Antibody was not detected in any pig in control non-infected pigs (group I) and in KV control pigs.

Serum neutralizing antibody

The serological response of pigs as measured by the SN test using PRRSV VR-2385 as an antigen is summarized in Fig. 3. Results are presented as the geometric mean ± one S.E.M. The SN antibody titer using PRRSV VR-2385 as an antigen was first detected in 7 pigs in group III on day 21, in 9 different pigs on day 28, and in 2 additional pigs on day 35. The geometric mean SN antibody titer was 0.35± 0.1 (n=20) on day 21, and increased to 3.9 ± 0.2 (n=18) on day 42. The SN antibody titer was first detected in 5 pigs in group IV on day 21, in 8 additional pigs on day 28, and in 3 different pigs on day 35. The geometric mean SN antibody titer was 0.27 ± 0.1 (n=17) on day 21, and increased to 2.6± 0.15 (n=15) on day 42. The geometric mean SN antibody titers in groups III and IV were similar from day 7 through day 35. However, the geometric mean SN titers of pigs in group III were significantly higher ($p \leq 0.05$) than the geometric mean SN titers of pigs in group IV on day 42. Neutralizing antibody was not detected in pigs of groups I and II.

The serological response of pigs as measured by SN test using PRRSV ISU-P as an antigen is summarized in Fig. 4. Results are presented as the geometric mean ± one S.E.M. Serum neutralizing antibody using PRRSV ISU-P as an antigen was first detected in group III in 7 pigs on day 28, 7 different pigs on day 35,

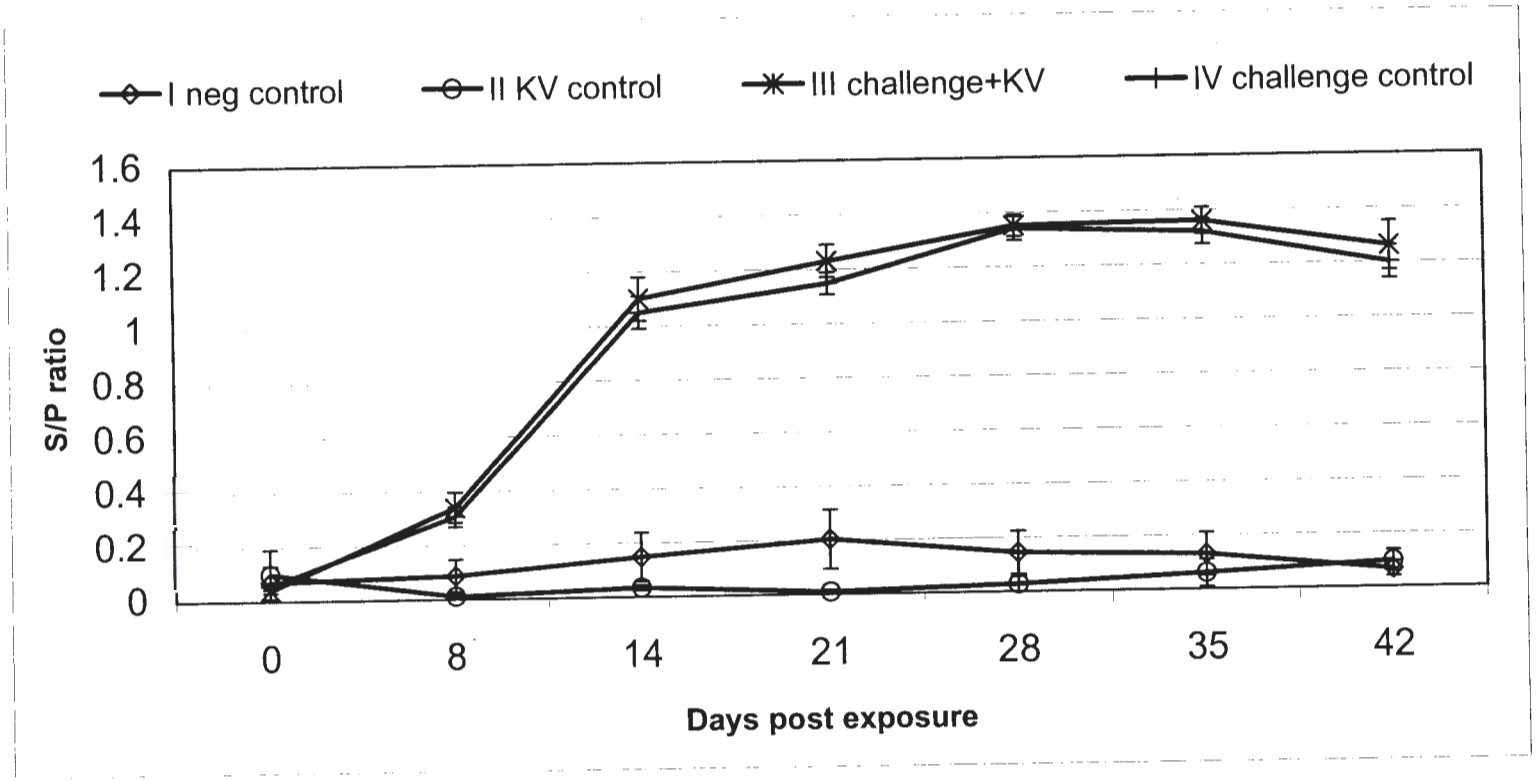

Fig. 2. Antibody response by ELISA for pigs in experiment 1, reported as an S/P ratio. An S/P ratio of ≥0.4 indicates a positive result. Results are represented as the geometric mean titer ± one S.E.M. Pigs were challenged with PRRSV VR-2385 on day 0. Total number of pigs on day 0 equals 5 in Groups I and II, and 20 in Groups III, and IV. One pig in group III was removed on days 28 and 35. Two pigs in Group IV were removed on day 14 and one pig was removed on days 21, 28 and 40.

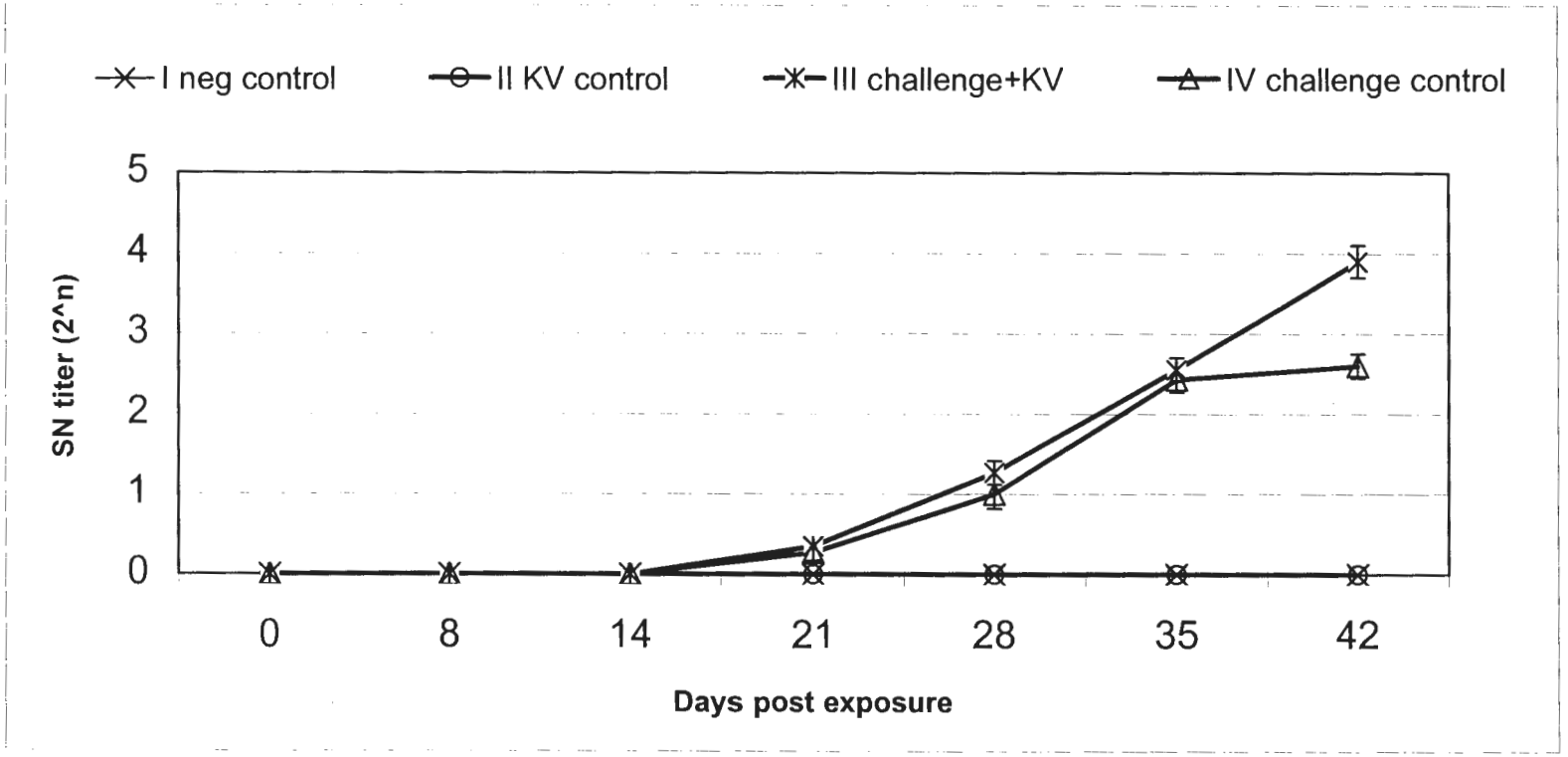

Fig. 3. Antibody response of pigs in experiment 1 by serum neutralization using PRRSV VR-2385. Results are represented as the mean titer ± one S.E.M. Pigs were challenged with PRRSV VR-2385 on day 0. Total number of pigs on day 0 equals 5 in Groups I and II, and 20 in Groups III, and IV. One pig in group III was removed on days 28 and 35. Two pigs in Group IV were removed on day 14 and one pig was removed on days 21, 28 and 40.

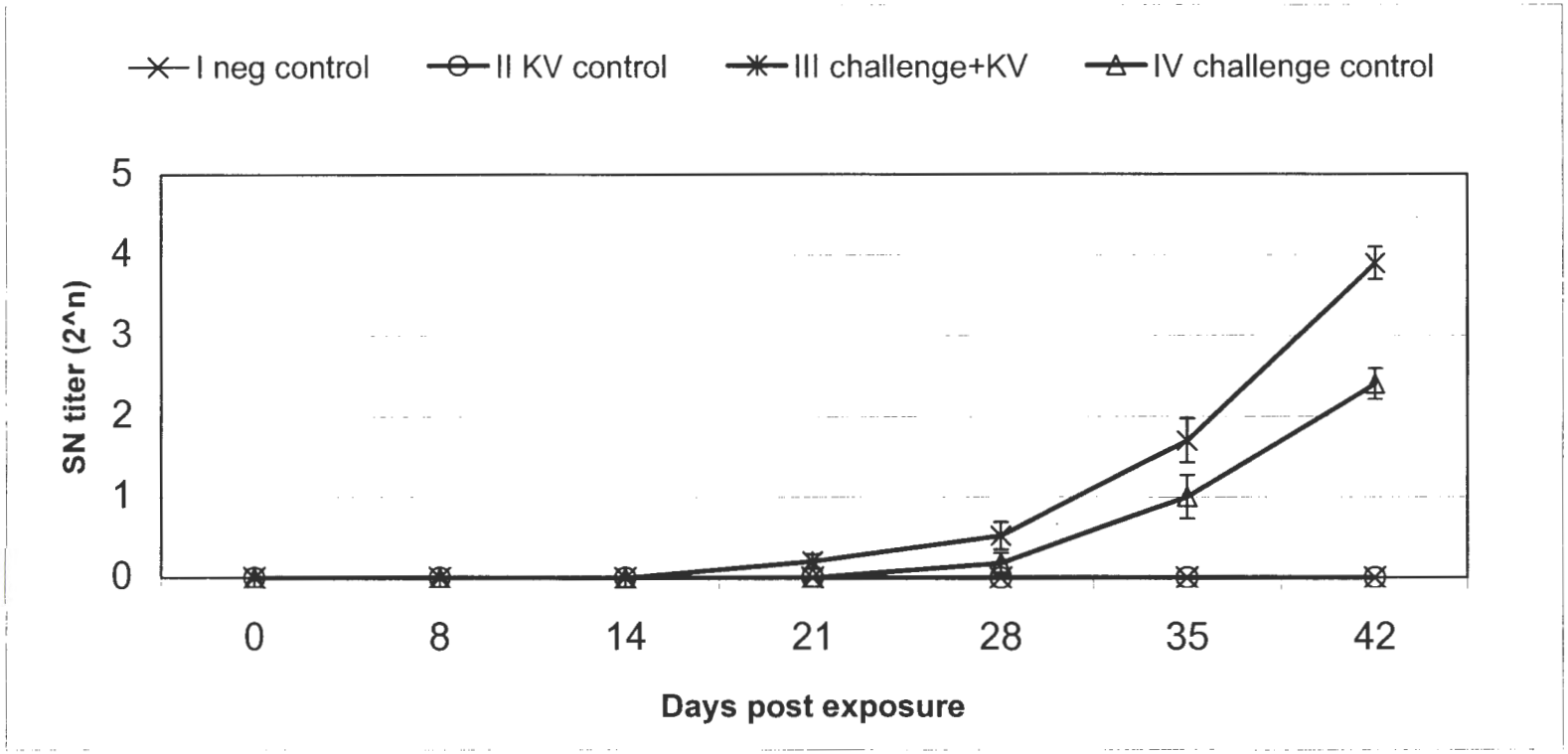

Fig. 4. Antibody response of pigs in experiment 1 by serum neutralization using PRRSV ISU-P. Results are represented as the mean titer ± one S.E.M. Pigs were challenged with PRRSV VR-2385 on day 0. Total number of pigs on day 0 equals 5 in Groups I and II, and 20 in Groups III, and IV. One pig in group III was removed on days 28 and 35. Two pigs in Group IV were removed on day 14 and one pig was removed on days 21, 28 and 40.

and 4 additional pigs on day 42. The geometric mean SN antibody titer was 0.2 $\pm$ 0.09 (n=20) on day 21, and increased to 3.9 $\pm$ 0.2 (n=18) on day 42. Serum neutraling antibody response was first detected in 3 pigs in group IV on day 21, in 1 different pig on day 28, and in 5 additional pigs on day 35. Serum neutraling antibody was not detected in 9 of 15 pigs on day 42. The geometric mean SN antibody titer was 0.18 $\pm$ 0.13 (n=16) on day 28, and increased to 2.4 $\pm$ 0.19 (n=15) on day 42. The SN titers in groups III and IV were similar from day 7 through day 35. On day 42, the geometric mean SN antibody titer of pigs in group III was significantly higher ($p \leq 0.05$) than the geometric mean SN antibody titer of pigs in group IV. Neutralizing antibody to PRRSV ISU-P was not detected in any pig in groups I and II.

Experiment 2

Response of pigs to PRRSV infection

Five pigs died during the study: 2 pigs in group III on day 7, 1 pig each in group IV on days 7 and 14, and 1 pig in group V on day 21. The cause of death was not determined. The ear tissues of all dead pigs were tested for porcine stress genes (Celera Genome, Davis, CA). Porcine stress genes were not detected.

The response of pigs to infection as measured by the duration of viremia is summarized in Table 1. Virus was first detected in all pigs in groups III, IV and V on day 7, and again in the same pigs on day 14. On day 21, virus was isolated in 5 of 6 pigs in group III, 6 of 7 pigs in group IV, and in all 8 pigs in group V. Virus was not detected in any pig in groups III, IV and V from day 28 through day 78.

The response of pigs to infection as measured by the titer of virus in serum is shown in Fig. 5. The mean virus titer in groups III, IV, and V reached maximum levels of $10^{4.26}$ $TCID_{50}$/ml, $10^{4.72}$ $TCID_{50}$/ml, and $10^{4.7}$ $TCID_{50}$/ml, respectively on day 14. The mean virus titer of all 3 groups then decreased to an undetectable level on day 28. Virus titers of pigs in groups III and IV were not different from that of pigs in group V. After the second challenge on day 65, virus was detected in all 4 pigs in groups I and II on days 68 and 71. Virus was not detected in any pigs of both groups on day 78. The virus titer in groups I and II reached maximum levels of $10^{3.9}$ $TCID_{50}$/ml, and $10^{3.6}$ $TCID_{50}$/ml, respectively on day 71. The mean virus titer of pigs in group I was not different from that of pigs in group II. Virus was not detected in any pig in groups III, IV and V after the second challenge.

Table 1

Proportions of viremic pigs (experiment 2)

	Days post exposure										
Group[a]	0	7	14	21	28	42	56	63	68	71	78
I	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	0/4
II	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	0/4
III[b]	0/8	8/8	6/6	5/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
IV	0/8	8/8	7/7	6/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
V	0/8	8/8	8/8	8/8	0/7	0/7	0/7	0/7	0/7	0/7	0/7

[a] Group I= negative control; Group II= killed virus vaccine (KV) control; Group III= challenged and KV on days 7 and 21; Group IV= challenged and KV on days 14 and 28; and Group V= challenged control. Total pigs = 4 in Groups I and II, and 8 in Groups III, IV and V. Five pigs died after sample collection. Two pigs in Group III died on day 7. One pig in Group IV died on days 7 and 14. One pig in Group V died on day 21.

[b] Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65.

The response of pigs to infection as measured by the duration of virus RNA in serum is summarized in Table 2. After the first challenge on day 0, virus RNA was first detected by PCR in serum of all pigs of groups III, IV, and V on day 7, and again in all pigs on days 14 and 21. Virus RNA was detected by PCR in 4 of 6 pigs in group III on day 28, and in the same 2 pigs on day 56. Virus RNA was detected by PCR in 4 of 6 pigs in group IV on day 28, but was not detected in any pig of group IV between days 42 and 63. Virus RNA was detected by PCR in 3 of 7 pigs in group V on day 28, and in 1 of these pigs on day 56. Virus RNA was not detected in the 6 remaining pigs between days 42 and 63. After the second challenge on day 65, virus RNA was first detected by PCR on day 68 in all pigs in groups I and II, and in the same pigs of both groups on days 71 and 78. Virus RNA was detected by PCR in 2 of 6 pigs in group III on day 78, and in 1 of 6 pig in group IV and 2 of 7 pigs in group V on day 68

No infectious virus was isolated from any oropharyngeal scraping collected from the pigs in groups III, IV, V. Virus RNA was detected by PCR in 4 of 6 pigs in group III on day 42, and in 2 of the same pigs on day 56. Virus RNA was detected by PCR in 2 of 6 pigs in group IV on day 42, and in the same 2 pigs on day 56. Virus RNA was detected by PCR in 2 of 7 pigs in group V on day 42, and in the same 2 pigs on day 56.

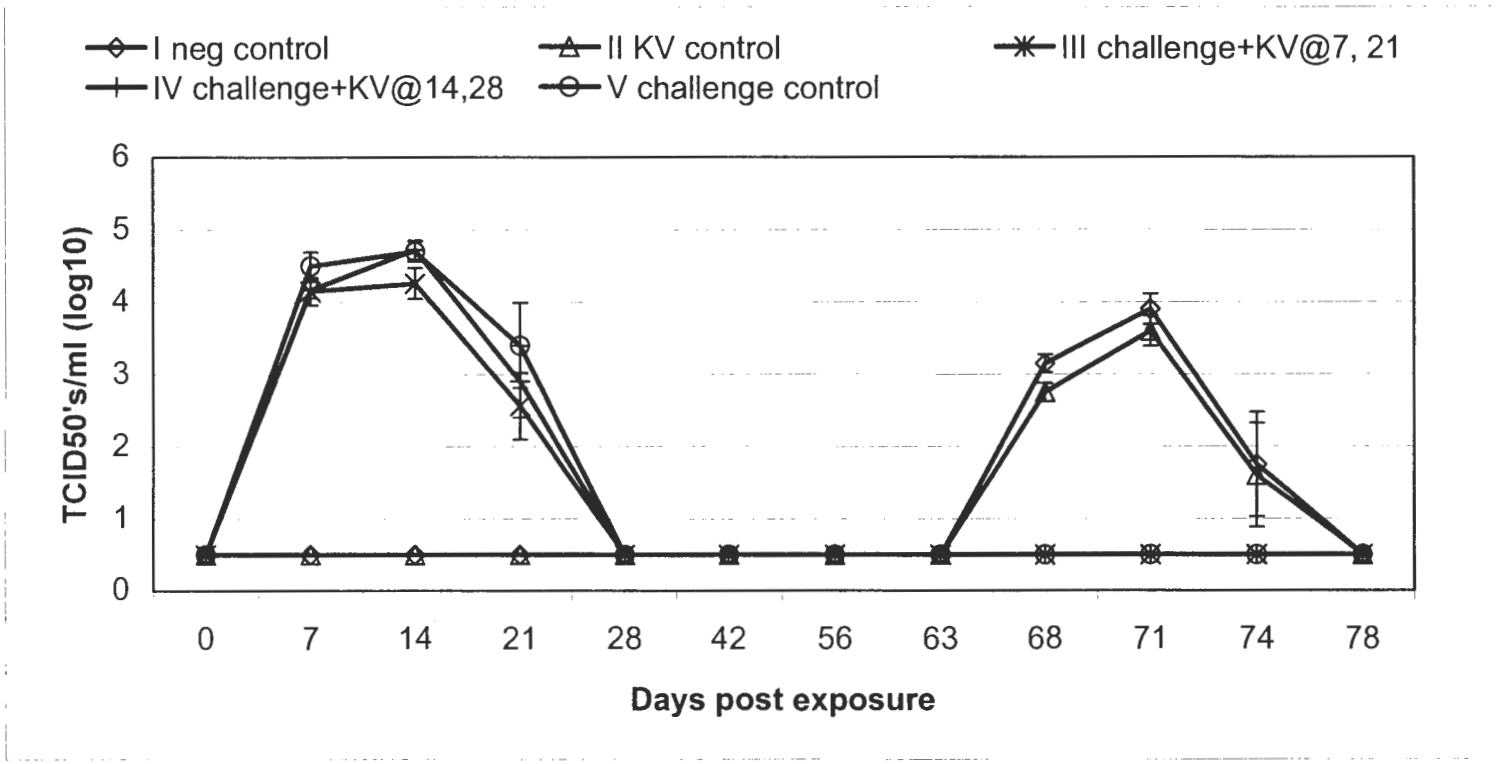

Fig. 5. Virus titer in serum of pigs in experiment 2, expressed as $TCID_{50's}$/ml (log_{10}). Results are represented as the mean titer $\pm$ one S.E.M. Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65. Total number of pigs on day 0 equals 4 in Groups I and II, and 8 in Groups III, IV and V. Two pigs in Group III died on day 7. One pig in Group IV died on days 7 and 14. Two pigs in Group V died on day 21.

Table 2
Proportions of pigs with detectable virus RNA in serum (experiment 2)

	Days post exposure										
Group[a]	0	7	14	21	28	42	56	63	68	71	78
I	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4
II	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	4/4
III[b]	0/8	8/8	6/6	6/6	4/6	0/6	2/6	0/6	0/6	0/6	2/6
IV	0/8	8/8	7/7	6/6	4/6	0/6	0/6	0/6	1/6	0/6	0/6
V	0/8	8/8	8/8	8/8	3/7	0/7	1/7	0/7	2/7	0/7	0/7

[a] Group I= negative control; Group II= killed virus vaccine (KV) control; Group III= challenged and KV on days 7 and 21; Group IV= challenged and KV on days 14 and 28; and Group V= challenged control. Total pigs = 4 in Groups I and II, and 8 in Groups III, IV and V. Five pigs died after sample collection. Two pigs in Group III died on day 7. One pig in Group IV died on days 7 and 14. One pig in Group V died on day 21.

[b] Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65.

Virus was detected in lung lavage samples collected at necropsy from 3 of 4 pigs in group I, and from 1 of 4 pigs in group II. Virus was not detected in any lung lavage sample of pigs in group III, IV, and V. Virus RNA was detected by PCR in all 4 pigs in group I and 2 of 4 pigs in group II. Virus RNA was detected by PCR in 3 of 6 pigs in group III, and in 4 of 6 pigs in group IV. Virus RNA was not detected in any pig of group V.

Virus was detected in tonsil tissues collected at necropsy of all 4 pigs in group I, and in 1 of 4 pigs in group II. Virus was not detected in tonsil tissues of any pig in group III, IV, and V. Virus RNA was detected by PCR in tonsil tissues of all 4 pigs in group I, of all 4 pigs in group II, and of all 6 pigs in group III. Virus RNA was detected by PCR in tonsil tissues in 5 of 6 pigs in group IV, and in 6 of 7 pigs in group V.

ELISA

The serological response of pigs as measured by ELISA is summarized in Fig. 6. Results are represented the mean $\pm$ one S.E.M. After the first challenge on day 0, antibody was first detected on day 14 in all pigs in groups III, IV, and V. The mean titers of groups III, IV, and V reached their highest levels of 1.47 ± 0.02, 1.18 ± 0.07, and 1.15 ± 0.07 on day 28, respectively. The mean titers then remained at or near those

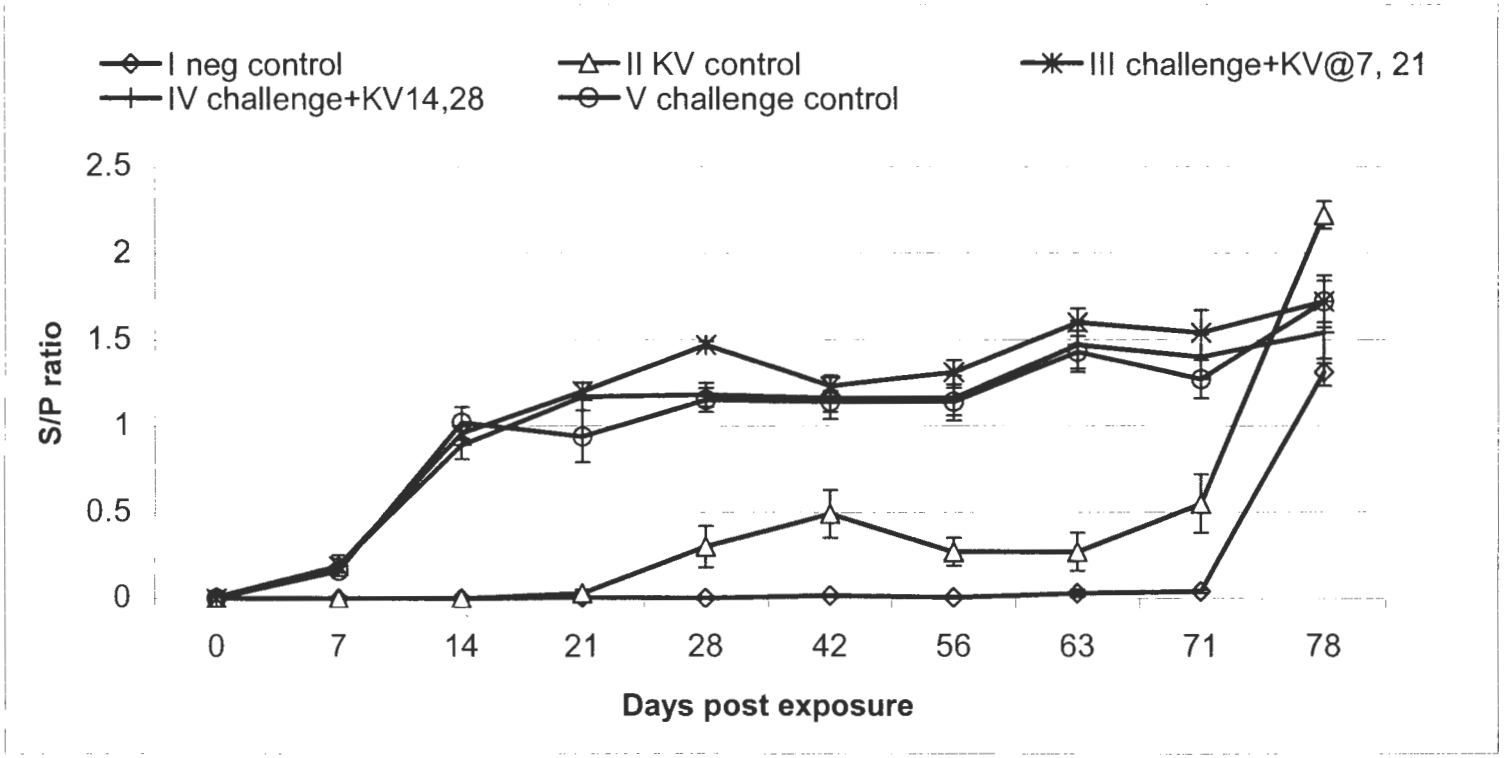

Fig. 6. Antibody response by ELISA for pigs in experiment 2, reported as an S/P ratio. An S/P ratio of ≥0.4 indicates a positive result. Results are represented as the mean titer ± one S.E.M. Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65. Total number of pigs on day 0 equals 4 in Groups I and II, and 8 in Groups III, IV and V. Two pigs in Group III died on day 7. One pig in Group IV died on days 7 and 14. Two pigs in Group V died on day 21.

levels from day 28 through day 78. The mean titers of groups III and IV were not different from that of group V. After the second challenge on day 65, antibody was detected in 2 of 4 pigs in group II on day 71, and the 2 remaining pigs on day 78. Antibody was first detected in all 4 pigs in group on day 78. The mean titer of the 4 pigs in group II were significantly higher ($p \leq 0.05$) than that of the 4 pigs in group I on day 78.

Serum-neutralizing antibody

The serological response of pigs as measured by the SN test using PRRSV VR-2385 as an antigen is summarized in Fig. 7. Results are represented as the geometric mean $\pm$ one S.E.M. Serum-neutralizing antibody to PRRSV VR-2385 was first detected in 4 of 6 pigs in group III on day 28. The SN antibody was detected in all pigs on day 42. The geometric mean SN antibody titer was 0.83 ± 0.34 (n=6) on day 28, and reached its highest level of 4.45 ± 0.54 (n=6) on day 63. The geometric mean SN antibody titer remained at or near this level, thereafter. The SN antibody was first detected in 4 of 6 pigs in group IV on day 28. Serum-neutraling antibody was detected in all 6 pigs on day 42. The geometric mean SN antibody titer was 0.25 ± 0.09 (n=6) on day 28, and reached the highest level of 3.08 ± 0.42 (n=6) on day 63. The geometric mean SN antibody titer remained at or near this level, thereafter. The SN antibody was first detected in 1 of 7 pigs in group V on day 28, 4 different pigs on day 42. The SN antibody was detected in all 7 pigs on day 56. The geometric mean SN antibody titer was 0.14 ± 0.14 (n=7) on day 28, and reached the level of 3.6 ± 0.49 (n=7) on day 63. The geometric mean SN antibody titer was maintained at or near this level, thereafter.

On day 28, the geometric mean SN antibody titer of pigs in group III was significantly higher ($p \leq 0.05$) than the geometric mean SN antibody titer of pigs in groups IV and V. The geometric mean SN antibody titers of pigs in groups III and IV were significantly higher ($p \leq 0.05$) than the geometric mean SN antibody titer of pigs in group V on days 42 and 56. The geometric mean SN antibody titers of groups III and IV were not different from the geometric mean SN antibody titer pigs in group V between days 63 and 78.

Serum-neutraling antibody was first detected in 1 pig in group II on day 71 and in the 3 remaining pigs on day 78. The mean SN antibody titer on day 78 was 0.91 ± 0.28 (n=4). Throughout experiment 2, no SN antibody was detected in any pig in group I. After the second challenge, the mean SN antibody titers of groups III, IV, and V slightly increased to the level of 4.1 ± 0.23 (n=6), 3.75 ± 0.38 (n=6), and 3.85 ± 0.4 (n=7), respectively.

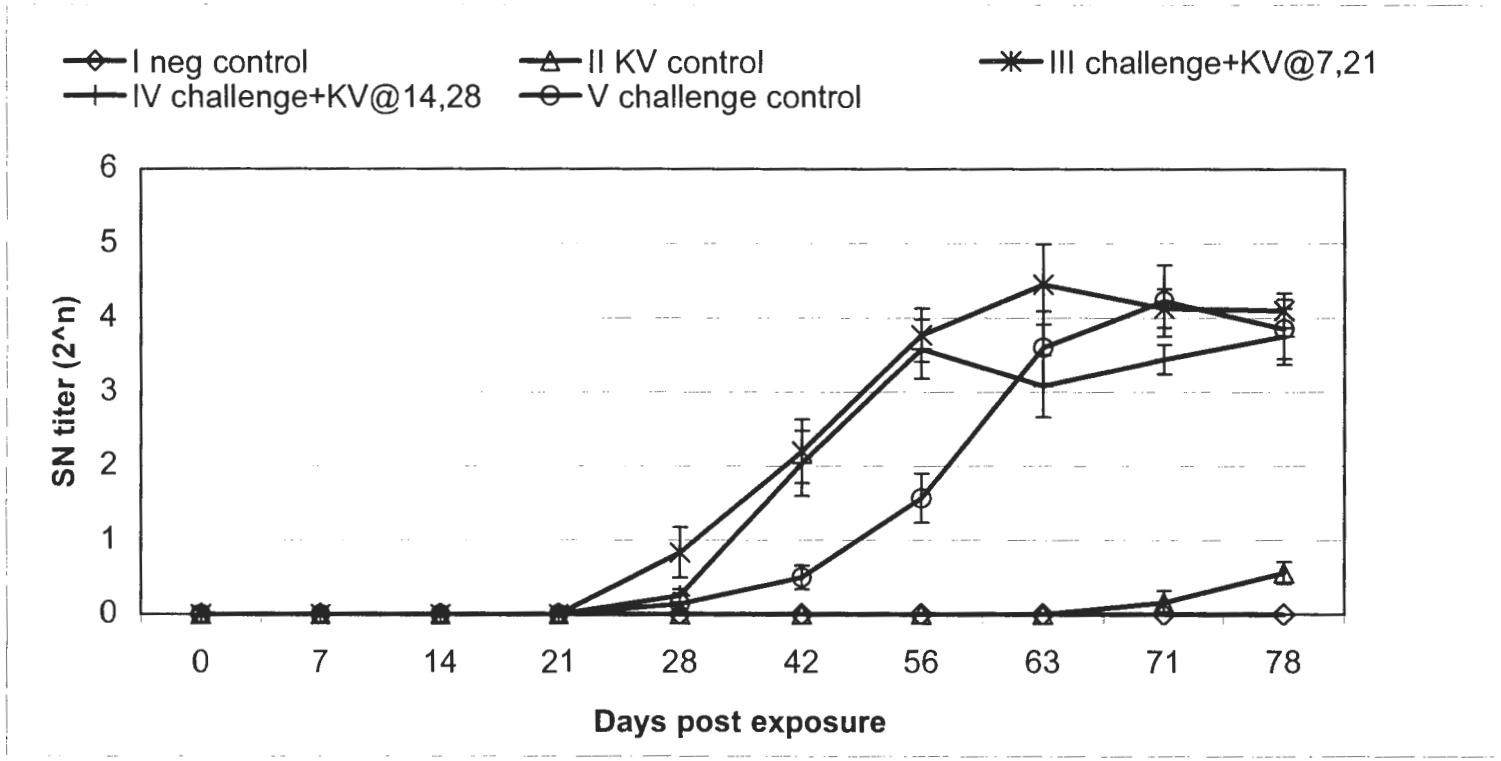

Fig. 7. Antibody response of pigs in experiment 2 by the serum neutralization test using PRRSV VR-2385. Results are represented as the mean titer ± one S.E.M. Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65. Total number of pigs on day 0 equasl 4 in Groups I and II, and 8 in Groups III, IV and V. Two pigs in Group III died on day 7. One pig in Group IV died on days 7 and 14. One pig in Group V died on day 21.

The serological response of pigs as measured by the SN test using PRRSV ISU-P as an antigen is summarized in Fig. 8. Results are represented the geometric mean ± one S.E.M. The SN antibody was first detected on day 28 in 5 of 6 pigs in group III, and in 1 remaining pig on day 35. The geometric mean SN antibody titer was 0.66 ± 0.21 (n=6) on day 28, and reached a maximum level of 4.33 ± 0.33 (n=6) on day 71. Serum-neutralizing antibody was first detected on day 42 in 5 of 6 pigs in group IV, and in 1 remaining pig on day 56. The mean SN antibody titer was 1.12 ± 0.47 (n=6) on day 42, and 3.37 ± 0.37 (n=6) on day 63. Serum neutralizing antibody was first detected on day 42 in 3 of 7 pigs in group V, and in 4 remaining pigs on day 56. The geometric mean SN antibody titer was 0.21 ± 0.11 (n=7) on day 42, and 3.46 ± 0.44 (n=7) on day 63.

On day 28, the geometric mean SN antibody titer of pigs in group III (0.66 ± 0.21) was significantly higher ($p \leq 0.05$) than the geometric mean SN antibody titer of pigs in groups IV and V, which ranged from 0 to 2. On day 56, the geometric mean SN antibody titer of pigs in groups II and III was significantly higher ($p \leq 0.05$) than the geometric mean SN antibody titer of pigs in group IV. On day 63 the titers of groups III and IV were similar to those of group V.

On day 78, the SN antibody was first detected in all 4 pigs in group II. The geometric mean SN antibody titer was 1.43 ± 0.35 (n=4). The SN antibody titer was not detected in any pig in group I. After the second challenge, the geometric mean SN antibody titers of groups III, IV, and V slightly increased to the level of 3.87 ± 0.45 (n=6), 4.12 ± 0.32 (n=6), and 4.21 ± 0.4 (n=7), respectively.

Cellular response of pigs as measured by the number of interferon gamma (IFN-γ) producing cells

The response of pigs as measured by the number of interferon gamma (IFN-γ) producing cells using PRRSV VR-2385 as an antigen is shown in Fig. 9. Results are represented as the mean ± one S.E.M. The mean number of IFN-γ producing cells of group III was 7.33±2.59 cells/ 5×10^5 lymphocytes (n=6) on day 28. It increased to 13.25±3.97 cells/5×10^5 lymphocytes (n=6) on day 42 and then decreased to 5.66±0.93 cells/ 5×10^5 lymphocytes (n=6) on day 63. After the second challenge, the number of cells increased to 9.41±1.86 cells/ 5×10^5 lymphocytes (n=6) on day 78. The number of IFN-γ producing cells of group IV was 3±0.9 cells/ 5×10^5 lymphocytes (n=6) on day 28, increased to 12.33±3.47 cells/ 5×10^5 lymphocytes (n=6) on day 42, and decreased to 5.25±2.29 cells/ 5×10^5 lymphocytes (n=6) on day 63. After the second challenge, the number of cells

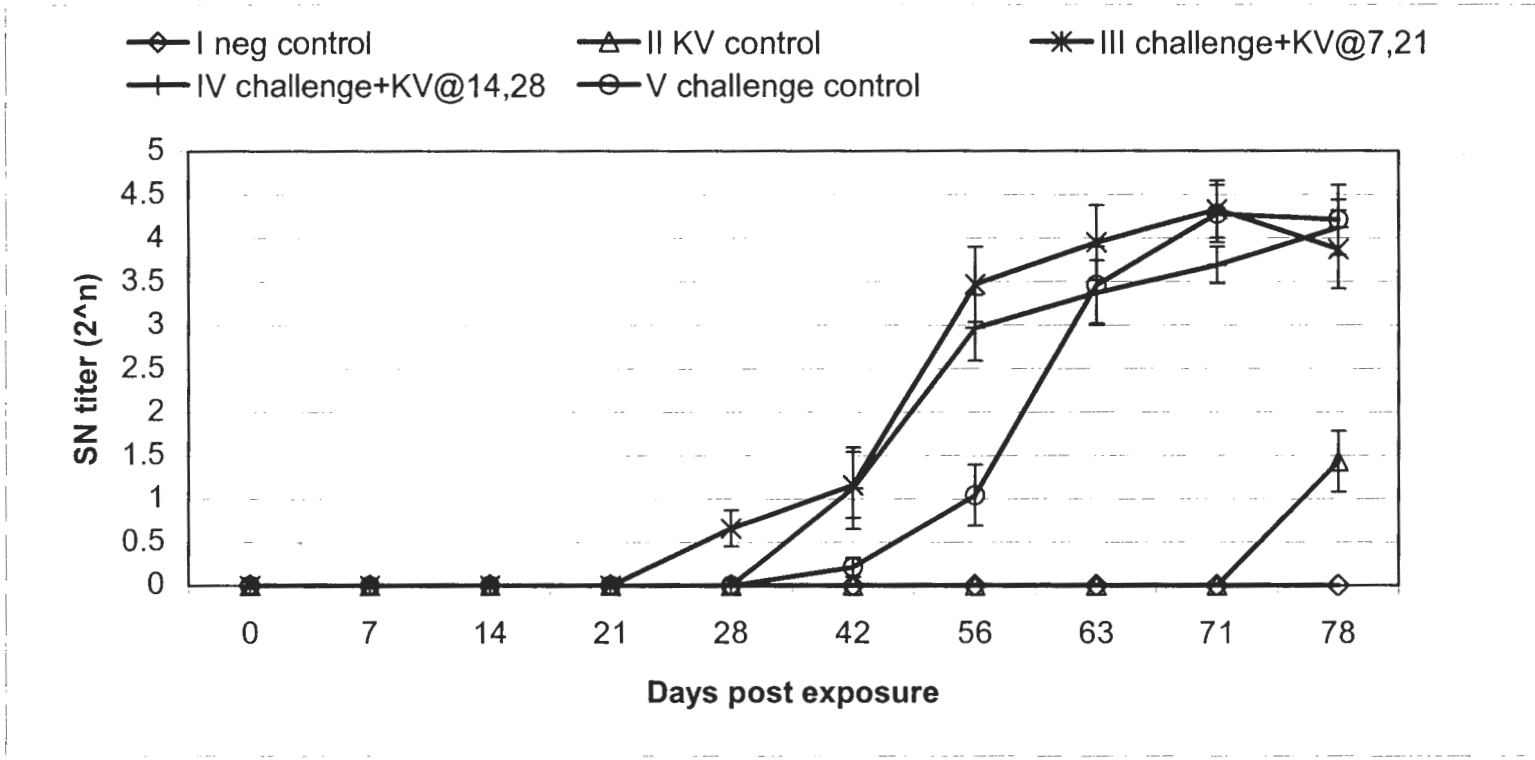

Fig. 8. Antibody response of pigs in experiment 2 by the serum neutralization test using PRRSV ISU-P. Results are represented as the mean titer ± one S.E.M. Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65. Total number of pigs on day 0 equals 4 in Groups I and II, and 8 in Groups III, IV and V. Two pigs in Group III died on day 7. One pig in Group IV died on days 7 and 14. One pig in Group V died on day 21

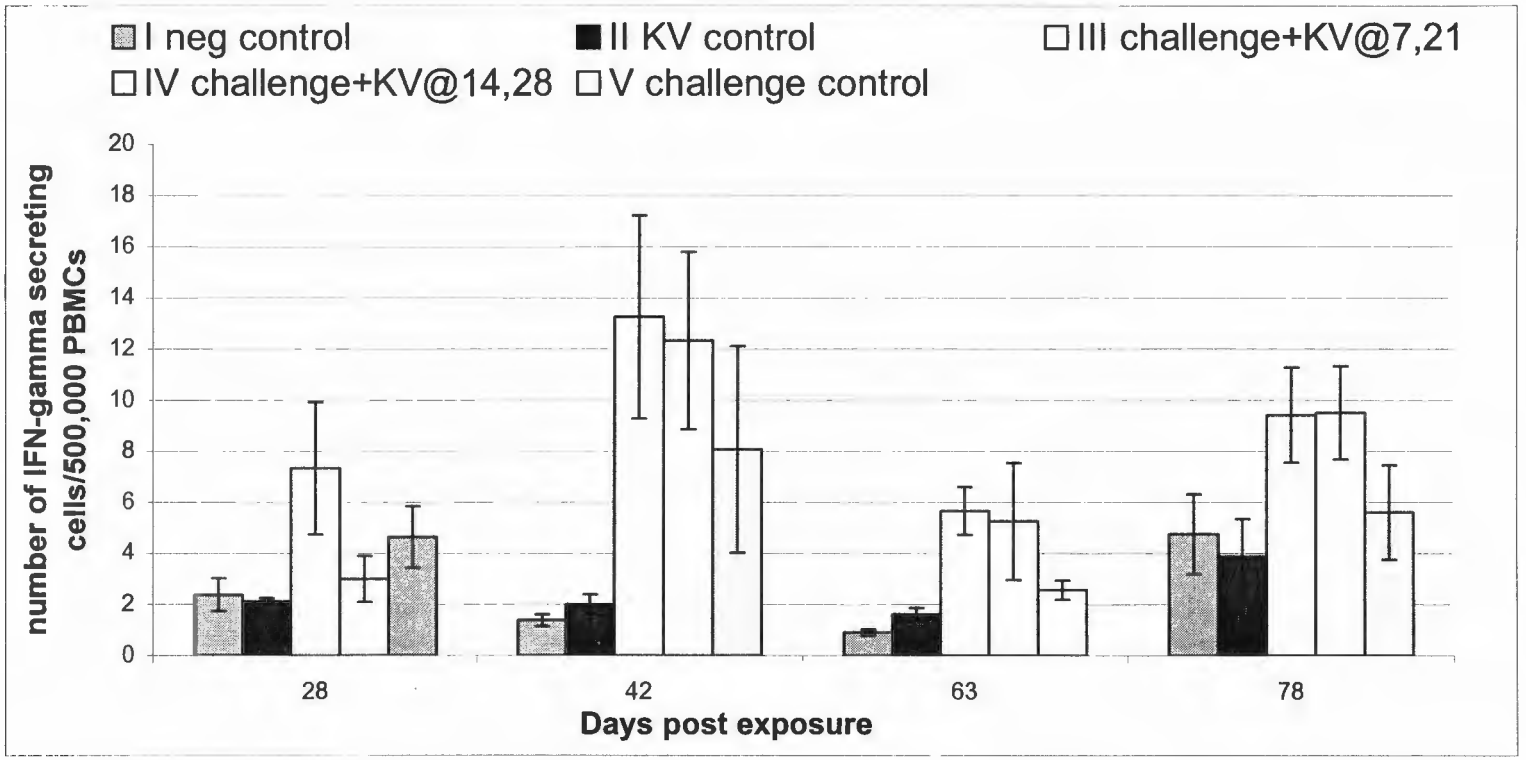

Fig. 9. Cellular response of pigs in experiment 2 as measured by numbers of IFN-γ producing cells of pigs using PRRSV VR-2385. Results are represented as the mean number of cells ± one S.E.M. Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65. Total number of pigs on day 0 equals 4 in Groups I and II, and 8 in Groups III, IV and V. Two pigs in Group III died on day 7. Two pigs in Group IV died on days 7 and 14. One pig in Group V died on day 21.

increased to 9.5±1.82 cells/ 5×10^5 lymphocytes (n=6) on day 78. The number of IFN-γ producing cells of group V was 4.64±1.2 cells/ 5×10^5 lymphocytes (n=7) on day 28, increased to 8.07±4.04 cells/ 5×10^5 lymphocytes (n=7) on day 42, and decreased to 2.57±0.38 cells/ 5×10^5 lymphocytes (n=7) on day 63. After the second challenge, the number of cells increased to 5.6±1.85 cells/ 5×10^5 lymphocytes (n=7) on day 78.

The response of pigs as measured by the number of IFN-γ producing cells using PRRSV ISU-P is shown in Fig. 10. Results are represented as the mean ± one S.E.M. Interferon gamma-gamma producing cellswere first detected in all pigs in all groups on day 28. The number of IFN-γ producing cells of group III was 20.83±2.42 cells/ 5×10^5 lymphocytes (n=6) on day 28, and maintained at a constant level between day 42 and day 63. After the second challenge, the number of cells decreased to 27.8±1.13 cells/ 5×10^5 lymphocytes (n=6) on day 78. The number of IFN-γ producing cells of group IV was 6.5±2.31 cells/ 5×10^5 lymphocytes (n=6) on day 28, increased to 41.16±8.92 cells/ 5×10^5 lymphocytes (n=6) on day 42, and decreased to 19.58±5.94 cells/ 5×10^5 lymphocytes (n=6) on day 63. After the second challenge, the number of cells increased to 4.83±0.83 cells/ 5×10^5 lymphocytes (n=6) on day 78. The number of IFN-γ producing cells of group V was 5.85±3.4 cells/ 5×10^5 lymphocytes (n=7) on day 28, increased to 9.85±1.36 cells/ 5×10^5 lymphocytes (n=7) on day 42, and decreased to 4.07±1.16 cells/ 5×10^5 lymphocytes (n=7) on day 63. After the second challenge, the number of cells was 5.5±1.4 cells/ 5×10^5 lymphocytes (n=7) on day 78.

The response of pigs as measured by the number of IFN-γ producing cells using 2 different strains of PRRSV suggests that the assay is not different by antigen used.

Discussion

The use of killed PRRSV vaccines to control PRRSV infection has been carried out. It is still unclear whether PRRSV KV products confer protective immunity against infectious PRRSV challenge. The KV products have reported to induce poor immune responses. However, the use of KV products in the field suggests that KV has the potential to confer protection under certain conditions. The killed PRRSV vaccines when given to pigs previously vaccinated with modified live PRRSV vaccines or when given to previously PRRSV infected pigs can induce grearter SN antibody response (Baker et al., 1999). Serum-neutralizing

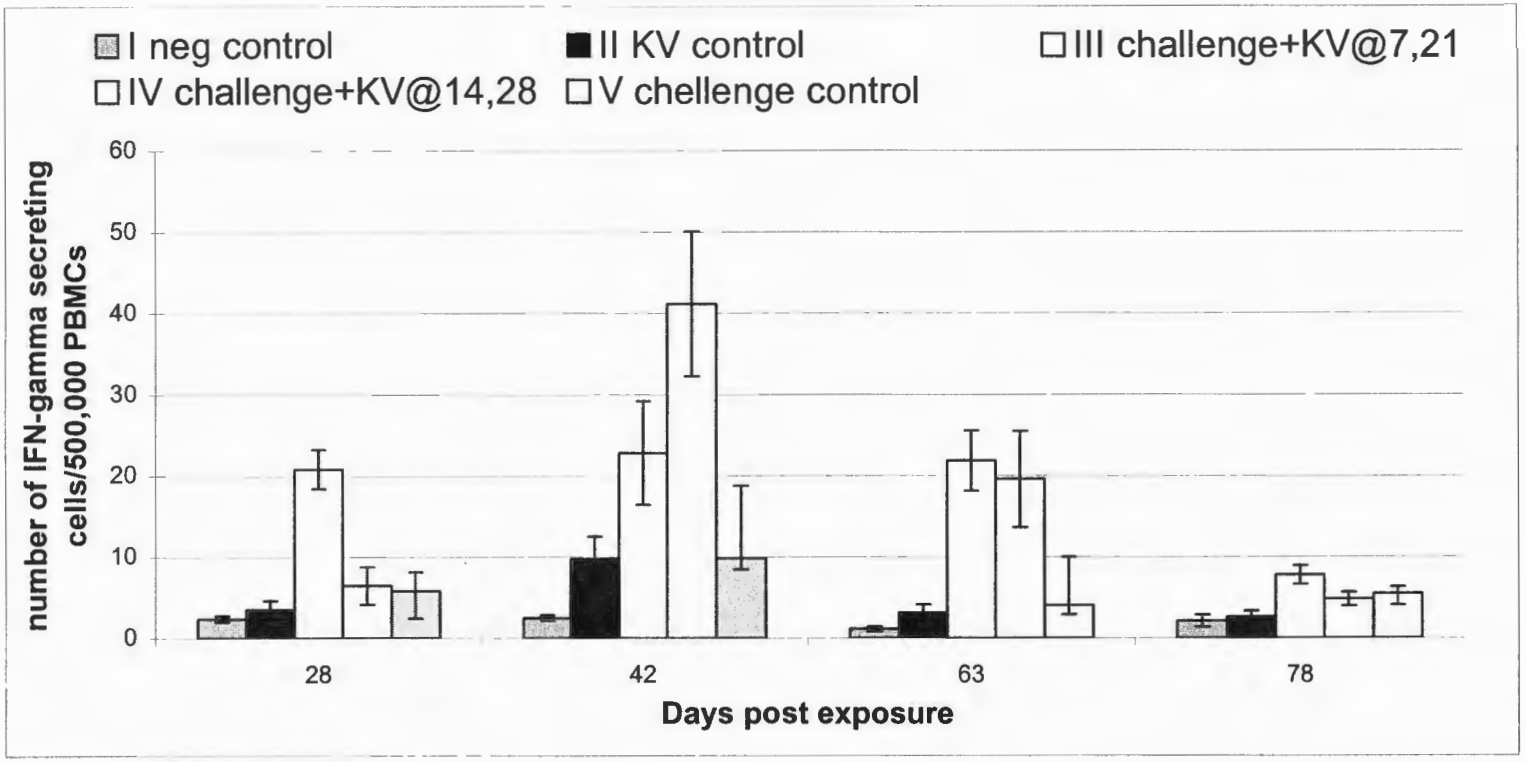

Fig. 10. Cellular response of pigs in experiment 2 as measured by numbers of IFN-γ producing cells of pigs using PRRSV ISU-P. Results are represented as the mean number of cells ± one S.E.M. Pigs in Groups III, IV and V were challenged with PRRSV VR-2385 on day 0, and pigs in all groups were challenged again with PRRSV strain 16244B on day 65. Total number of pigs on day 0 equals 4 in Groups I and II, and 8 in Groups III, IV and V. Two pigs in Group III died on day 7. Two pigs in Group IV died on days 7 and 14. One pig in Group V died on day 21.

antibodies have recently been identied as a key component of the protective immunity against PRRSV infection (Joo et al., 1999; Osorio et al., 2001). If KV has the ability to reduce PRRSV shedding through the induction of immune response, it could potentially be used to shorten the acclimatization period for gilt introduction in positive farms. This study was designed to determine whether PRRSV shedding of pigs previously infected with virulent PRRSV could be reduced by the use of a commercial KV.

Two experiments were conducted using a commercial KV that was manufactured using PRRSV ISU-P. This PRRSV strain was different from PRRSV VR-2385 that was used to challenge pigs on day 0. In the first experiment, infected pigs were treated with KV on 14 and 28 days p.i. Viremias of KV-treated and non-treated infected groups were compared between 0 and 42 days p.i. The second experiment was conducted in an identical manner to the first experiment with the following exceptions. Two vaccine treatment groups were used. One group of infected pigs was treated with the KV on 7 and 21 days p.i., the other group of infected pigs was treated with KV on 14 and 28 days p.i. Oropharyngeal scrapings collected on days 42 and 56 p.i. were tested for virus and virus RNA. In both experiments, there was no significant difference in magnitude and duration viremias of KV-treated infected pigs and non-treated infected pigs. In the second experiment, virus RNA was only detected in 2 of 6 KV-treated infected pigs, and in 2 of 6 non-treated infected pigs.

The results of these experiments indicate that a commercial KV treatment has no effect on virus shedding in pigs infected with a heterologous strain of PRRSV. Previous experiments to test the effect of vaccine on virus shedding were done by Swenson et al. (1995) and Nielsen et al. (1997). Swenson et al. (1995) found that KV had an effect on shedding. In their study, the PRRSV strains used for vaccination and challenge were the same. These results were not duplicated by Nielsen et al. (1997). These investigators found that KV had no effect on virus shedding. Virus used to produce the vaccine and challenge strain was heterologous. Furthermore, these investigators observed a reduction in virus shedding was observed in pigs treated with MLV. The observation that virus shedding was reduced in MLV-treated pigs suggests that a component of the cell-mediated immune (CMI) response plays an important role in controlling PRRSV infection. The apparent reduction of virus shedding in semen observed by Swenson et al. (1995) may have been due to the induction of CMI component by their KV or protection that was induced by the same strain.

Lager et al. (1997a, 1997b) reported that gilts exposed to PRRSV isolate NADC-8 at the time of breeding (day 0) were completely protected against subsequent homologous challenge on day 90 and the protection was at least 604 days after initial exposure. In contrast, heterologous protection has been shown to be incomplete and may have a shorter duration than homologous protection (Lager et al., 1999). These observations suggest that protection might be dependent upon the antigenic similarity between the immunizing and challenge viruses (Van Woensel et al., 1998). In addition, a study by Benson et al. (2000) also suggested that protection against reinfection by heterologous strains might be challenge-dose dependent. In the current study, the challenge virus and vaccine virus were heterologous, and the challenge dose was considered a high amount ($10^{5.4}$ $TCID_{50}$/ml).

In the current study, in order to measure protective immunity, all pigs in experiment 2 were challenged with PRRSV isolate 16244B. This was done to mimic field conditions and possible scenarios that would reflect re-exposure in commercial farms. The presence of viremia was used to determine the efficacy of protective immunity. Interestingly, the PRRSV isolate 16244B was not recovered from KV-treated infected pigs, and non-treated infected pigs. Attempts to isolate the virus from lung lavage and tonsil samples also failed to recover the virus. However, PCR was able to detect the presence of virus RNA in some serum samples. Similar results were reported by Joo et al. (1999) and Direksin et al. (2000). The presence of specific antibodies alone might not be enough to protect pigs from re-infection or from viremic. The CMI component might protect pigs from reinfection as well.

Serum neutralizing antibody response was not observed in PRRSV-negative pigs that were vaccinated with KV. In contrast, pigs previously infected with PRRSV responded immunologically to the KV treatment. Increases were observed in SN titer over time and in the numbers of IFN-γ producing cells. The geometric mean SN antibody titer of the KV-treated group was significantly greater ($p \leq 0.05$) on day 42 than that of the non-treated groups in the first experiment (Figs. 3 and 4), and on days 42 and 56 in the second experiment (Figs. 7 and 8). In the first experiment, the geometric mean SN antibody titer in KV-treated and non-treated infected groups was 3.9$\pm$ 0.2 and 2.6$\pm$ 0.15, respectively on day 42. In the second experiment, the mean SN antibody titer in 2 KV-treated and 1 non-treated infected groups was 2.3$\pm$ 0.23 and 0.5$\pm$ 0.16 on day 42, and 3.67$\pm$ 0.38 and 1.57$\pm$ 0.33, respectively, on day 56. These observations indicate that the KV treatment of previously

infected pigs increased in the humoral immune response as indicated by the SN antibody titer. Similar increases in antibody titer were reported by Baker et al. (1999) in previously MLV-vaccinated sows after vaccination with KV. In the aforementioned study, sows were obtained from herds that used PRRSV MLV routinely and were either vaccinated with two doses of KV or had experienced repeated MLV vaccination at the time of the second dose of KV. Following vaccination, only the pigs in the KV vaccination groups had increased SN antibody titer, with a group geometric mean titer of 3 to 8.5. On the other hand zero to minimal increase in SN titer was observed in pigs vaccinated with MLV. Similar results were obtained by Trayer (1999) after KV vaccination. Sows in endemically infected herds having titers less than 1:16 were identified as "at risk". In order to verify if the use of KV can reduce the "at risk" population, the sows were vaccinated with two doses of KV. Serum-neutralizing antibody response was monitored a month later. The use of KV reduced the "at risk" population. These results imply that previously infected sows had increased SN antibody after KV vaccination. Increased SN antibody was also observed when previously MLV-vaccinated pigs were vaccinated with KV (Joo et al., 1999). These pigs were vaccinated with MLV at day 0, and re-immunized with KV and MLV at day 35 and 53, respectively. From days 35 to 53, increasing SN titers were observed, however, the experiment lacked a control group that would indicate if the response were from KV or the first MLV vaccination. An increase in SN antibody was also reported when challenging previously infected pigs with a homologous strain of virus at 77 days post first exposure, and it increased nearly twofold after the second exposure (Shibata et al., 2000).

Pigs infected with PRRSV VR-2385 that were treated with KV during days 42 to 63 also had higher numbers of IFN-γ producing cells than infected pigs not treated with KV. However, the difference was not statistically significant. The number of PRRSV specific IFN-γ producing cells persisted only a few weeks. The increased level might be due to anamnestic response or polyclonal activation of PBMCs. Previous studies reported that the response should have been maintained at a high level for an extended period. A study by Meier et al. (2000) found that IFN-γ producing cells gradually increase after detection, which takes approximately 8 to 10 weeks p.i. The number of IFN-γ producing cells reaches its highest level on day 300 and maintains this level up to 600 days p.i (Meier et al., 2000). However, the results of the current study were more similar to findings by Bautista and Molitor. (1997), in which previously infected pigs developed anamnestic response following re-exposure to the homologous strain. The anamnestic response was higher in magnitude than non-reexposure

pigs, yet it remained for a couple of weeks. In that study, previously infected pigs were re-challenged with the homologous strain at 20 weeks post first exposure. An anamnestic response was observed two weeks later, and then it declined through week 24.

The observations by Swenson et al. (1995) and Nielsen et al. (1997) suggest that the apparent reduction of virus shedding in semen might be due to a component of CMI response or homologous protection. Future efforts to reduce virus shedding by vaccine treatment should focus on vaccination protocols and vaccines that increase mucosal immunity, or the use of autogenous homologous KV.

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CHAPTER 5. GENERAL CONCLUSIONS

General Conclusions

The studies described in this thesis were designed to: (a) compare the shedding patterns of wild-type and vaccine viruses; and (b) determine if virus shedding in pigs previously infected with virulent porcine reproductive and respiratory syndrome virus (PRRSV) could be significantly reduced or inhibited by enhancing the immune response through the use of a commercial killed vaccine (KV). A shorter virus-shedding period would decrease the acclimatization period of pigs before introduction into a herd.

The study described in Chapter 2 was designed to compare the virus shedding pattern of pigs infected with wild-type (PRRSV VR-2332) or vaccine virus (PRIME PAC™, Schering-Plough). Two groups of 8 pigs were infected intramuscularly with wild-type or vaccine PRRS virus. Pigs in all groups were monitored for virus shedding over a 105-day period. Oropharyngeal scrapings were collected weekly and assayed for virus and virus RNA beginning on 49 days post infection (p.i.). Sentinels were also used as biological indicators to detect virus transmission. They were placed in contact with principal pigs on days 77 and 91. Virus was not isolated from any oropharyngeal scraping of any pig infected with vaccine or wild-type virus. In contrast, virus RNA was detected in oropharyngeal scrapings of pigs between 49 and 70 days p.i. that were infected with vaccine virus, and in oropharyngeal scrapings of pigs between 70 and 105 days p.i. that were infected with wild-type virus. Virus RNA was detected in tonsils at necropsy on day 109 in 3 of 6 pigs infected with vaccine virus and in 2 of 6 pigs infected with wild-type virus. Sentinel pigs did not seroconvert as indicated by ELISA. The presence of virus RNA in tonsils of both groups at necropsy suggests that virus remained in pigs, but could not be transmitted to sentinel pigs. Whether virus shedding can be reinitiated needs to be further evaluated.

It is interesting to note that the difference between the duration of vaccine virus shedding described by Srinivasappa J. et al. (1999) and the duration of wild-type virus shedding reported by other investigators (Albina et al., 1994; Lager and Mengeling, 1996; Terpstra et al., 1992; Wills et al., 2000) ranged from 20 to 30 days. This difference in shedding patterns of wild-type and vaccine viruses corresponds to the difference in the number of days that virus RNA was detected in oropharyngeal scrapings of pigs infected with wild-type or vaccine virus. The detection of virus RNA is not necessarily equated to the isolation of infectious virus. However, the difference in the detection rate of virus RNA in wild-type and vaccine viruses in oropharyngeal

scrapings does suggest that vaccine virus is shed for a shorter period of time than wild-type virus. These observations suggest that immunization with vaccine virus in the acclimatization process prior to the introduction of replacement pigs into a herd might shorten the length of the acclimatization period as compared to exposure to wild-type virus.

In this study, there was relatively small number of pigs in each group, and only 1 strain of wild-type and vaccine viruses were evaluated. Thus, additional experimentation involving different wild-type and vaccine virus strains needs to be done before a definitive conclusion can be made.

The study described in Chapter 3 was designed to determine if virus shedding of pigs previously infected with virulent PRRSV VR-2385 could be reduced by enhancing immune responses through the use of a commercial KV (PRRomiSe®, Intervet Inc.). Two experiments were conducted. In the first experiment, 2 groups of 5 non-infected control pigs and 2 treatment groups of 20 infected pigs each were used. One group of infected pigs was treated with KV on 14 and 28 days p.i. The other group of infected pigs was not treated with KV. Sera were collected weekly between 0 and 42 days p.i., and assayed for virus and antibody. The viremias of KV-treated infected pigs and non-treated infected pigs were compared. The second experiment was conducted in an identical manner to the first experiment with the following exceptions. The number of pigs per group was reduced to 8. Two vaccine treatment groups were used. One group was treated with KV on 7 and 21 days p.i., and the second group was treated with KV on 14 and 28 days p.i. Oropharyngeal scrapings were tested for virus and virus RNA on day 42 and 56 following virus exposure. There was no significant difference in the virus shedding pattern between KV- treated infected pigs and non-treated infected pigs. The viremia of KV-treated infected pigs and non-treated infected pigs lasted for 21 and 28 days, respectively, in the first experiment. In the second experiment, the viremias of both KV-treated infected pigs and non-treated infected pigs lasted for 28 days. The magnitude of viremia of KV-treated infected pigs was not significantly different from that of non-treated infected pigs. No virus was isolated from oropharyngeal scrapings of any pig in any group in the second experiment, nor was there a significant difference between treatment groups in the detection of virus RNA. Virus RNA was detected in 2 of 6 infected pigs treated with KV, and in 2 of 6 infected pigs that were not treated with KV. These observations indicate that KV treatment has no effect on virus shedding.

Early attempts to test the effect of KV on virus shedding were done by Nielsen et al. (1997) and Swenson et al. (1995a). The results were conflicting. Swenson et al (1995a) found infectious virus in semen of 2 of 4 KV-treated pigs only on days 4 and 7. Infectious virus was detected periodically in the 2 remaining pigs between 4 and 28 days p.i. In contrast, infectious virus was detected continuously in semen of all 3 non-treated pigs between days 4 and 32 p.i. These observations suggest that KV has an effect on shedding. The results by Swenson et al. (1995a) were not duplicated by Nielsen et al. (1997), who conducted a similar study to evaluate if the use of KV or modified-live vaccine (MLV) could reduce virus shedding in semen. These investigators found that KV had no effect on virus shedding. However, the investigators observed a reduction in virus shedding in pigs treated with MLV. The observation that virus shedding is reduced in MLV-treated pigs suggests that cell-mediated immunity (CMI) plays an important role in controlling PRRSV infection. The apparent reduction of virus shedding in semen observed by Swenson et al. (1995a) may be due to the induction of a CMI component by their KV and the homologous protection. An apparent reduction of virus shedding in semen may be due to a component of CMI response. The treatment of pigs with KV in the present study also appears to enhance a component of CMI response, as indicated by the apparent increase of IFN-γ producing cells. However, virus shedding in semen is not necessarily associated with virus shedding from mucosal surfaces such as the upper respiratory tract. Therefore, future efforts to reduce virus shedding by vaccine treatment should focus on vaccines that increase mucosal immunity, and CMI.

Although KV treatment had no effect on virus shedding, infected pigs responded immunologically to the KV treatment. In the experiments, increases were observed in the SN antibody titer over time (Experiments 1 and 2) and in the number of IFN-γ producing cells (Experiment 2). The mean SN antibody titer of the KV-treated infected group was significantly greater ($p \leq 0.05$) than that of the non-treated infected group on day 42 in the first experiment, and on days 42 and 56 in the second experiment. The geometric mean SN antibody titer of the KV-treated infected group was similar to that of the non-treated infected group from 0 to 35 days p.i. in the first experiment, and from 0 to 28 and from 63 to 78 days p.i. in the second experiment. Antibody responses as determined by SN and ELISA tests were observed in control non-infected pigs vaccinated only with 2 doses of KV.

In the second experiment, it is interesting to note that the mean SN antibody titers of the KV-treated infected groups were similar to that of the non-treated infected groups after 63 days p.i. The reason could be due to the antibody feedback mechanism in which antibody can inhibit normal antibody formation during conventional antibody response. The mean number of PRRSV specific IFN-γ producing cells also increased between days 28 and 36 in the KV-treated infected groups, as compared to non-treated infected group. These observations suggest that recovered pigs can restore immunity after diminution during early PRRSV infection, which concur with observations by Vezina et al. (1996) and Lopez Fuertes et al. (1999).

In addition, pigs in the study described in Chapter 3 were monitored to determine if SN antibodies are protective. All pigs were challenged with another virulent heterologous PRRSV (strain 16244B) on day 65, when the mean SN antibody titer appeared to reach or approximate the maximum level. Infectious virus was not detected in serum of any pigs in either the KV-treated infected groups or the non-treated infected group following challenge with heterologous PRRSV. The results suggest that pigs with neutralizing antibody are protected against subsequent challenge with heterologous PRRSV, as observed by the reduction of viremic pigs. Similar results were reported by Joo et al. (1999) and Osorio et al. (2001). In both studies, virus was not detected in any sow having SN antibodies either by natural infection or passive transfer following infection.

The present studies provide useful information that reinforces temporary herd closure as well as the acclimatization process. As previously indicated, temporary herd closure consists of closing a herd for an extended period of time before restocking and acclimating replacement pigs prior to introduction to allow the development of specific immunity and cessation of virus shedding. The results of the present study suggest that pigs exposed to vaccine virus shed the virus for a shorter period of time than pigs exposed to wild-type virus. It appears that immunization with vaccine virus in the acclimatization process prior to the introduction of replacement pigs into a herd might shorten the length of the acclimatization period. Even though the use of KV does not reduce virus shedding in pigs previously infected, pigs previously infected with virulent PRRSV responded immunologically to the KV treatment as determined by a significant increase in the level of SN antibody and an insignificant increase the number of IFN-γ producing cells. The use of KV could help to increase protective immunity of infected pigs during herd closure as well as acclimatization.

Future Research

The complexity regulating the immune response of pigs to porcine reproductive and respiratory syndrome virus (PRRSV) infection is clearly indicated by the absence of a virus-inhibitory function such as serum neutralizing (SN) antibodies and interferon-gamma (IFN-γ) producing cells during the first few weeks following infection. The specific SN antibodies and IFN-γ producing cells to PRRSV infection become detectable several weeks following virus exposure (Meier et al., 2000b). Despite the appearance of non-neutralizing antibodies as early as a week p.i., these antibodies are not associated with virus protection or an inhibitory function. A delay in virus-inhibitory response is observed in pigs infected with wild-type virus, as well as pigs vaccinated with the currently available PRRSV modified-live vaccine (MLV). It is suggested that the delayed SN antibody response is possibly due to the effect of PRRSV in modulating the host's immune response. The effect of PRRSV infection on the host's immune system also appears to be bivalent, which could be either immunosuppressive or immnoenhancing depending on the phase of infection (Vezina et al., 1996). During the first few weeks after exposure, PRRSV appears to suppress the host's immune system, as demonstrated by the absence of SN antibodies and IFN-γ producing cells. Suppression appears to be overcome later by the host because SN antibodies and IFN-γ producing cells become detected. The reason for a delayed PRRSV-inhibitory immune response is unclear. However, there is evidence suggesting possible regulation by a cytokine such as interleukin (IL) -10 (Thanawongnuwech et al., 2001).

Accessory cell-derived cytokines such as IL-4, IL-10, and IL-12 are able to suppress and enhance the intensity of virus-specific cellular immunity in vitro. Thanawongnuwech et al. (2001) observed a cytokine profile following PRRSV infection characterized by an increased level of IL-10 mRNA transcript from pulmonary alveolar macrophages (PAMs) 28 days following virus exposure. In contrast, IL-12 mRNA transcript from PAMs was not observed during the study. An increased level of IL-10 might be associated with an undetectable level of IFN-γ producing cells in early PRRSV infection. Interleukin-10 has been shown to suppress macrophage activation, to direct the differentiation of Th0 cells to Th2 cells and to inhibit the generation of Th1 cells (Abbas et al., 1997). Therefore, the production of IFN- producing cells is suppressed.

The currently available PRRSV MLV vaccine (RespPRRS/Repro™) provides a sub-optimal level of protective immunity. There is also controversy regarding the effectiveness of this vaccine in the field. A study

by Meier et al (2000b) indicated that PRRSV MLV does not stimulate a strong cellular immunity or induce a significant response of SN antibodies. Therefore, ability to manipulate a vaccine-induced immune response in the direction of a predominant CMI should be investigated to develop a more effective vaccine against PRRSV infection.

The quality of immune response induced by a vaccine can be modulated by the type of adjuvant used in the vaccine. The use of a cytokine as an adjuvant will modulate immune response. Interleukin-12 is one of the cytokines currently being examined for this purpose. As such, it has attracted attention as a possible adjuvant due to its known ability to stimulate CMI. This cytokine is known to direct the differentiation of T-cells into IFN-gamma producing cells following their exposure to antigens. Interleukin-12 has also been shown to promote the development of protective immunity against several pathogens (Zuckermann et al., 1998) Thus, it has potential as a vaccine adjuvant.

Another possibility, in addition to the administration of exogenous IL-12, is concurrent vaccination with PRRSV and Pseudorabies (PRV) MLVs. A study by Meier et al. (2000b) reported that PRV MLV is capable of generating a better immune response than PRRSV MLV. The specific SN antibodies and IFN-γ producing cells induced by PRV MLV are detected only a week after vaccination, and reach the highest level within 2-3 weeks post vaccination. On the other hand, reaching this maximum level takes 9-10 months following PRRSV MLV vaccination. In the priming of a CMI response, Th0 cells would be directed towards the differentiation of Th1 cells that favor the induction of CMI response. Consequently, IL-12 and IFN-γ would be secreted more. The hypothesis would be that concurrent vaccination with PRRSV and PRV MLVs induces a greater CMI response against PRRSV infection than vaccination with PRRSV MLV alone.

An interesting observation in the present studies is that the use of KV in previously infected pigs tends to enhance SN antibody response and the quantity of IFN-γ producing cells. However, when given alone to non-infected pigs, KV has no effect on the induction of an immune response. These observations lead to the hypothesis that the administration of an epitope responsible for SN antibody response in conjunction with the presence of IFN-γ producing cells in the host results in an increase in SN antibody response and, subsequently, increases protective immunity of the host. An epitope responsible for SN antibody could be administrated by a commercially available KV, autogenous vaccine, or subunit vaccine. In the present studies, a commercial KV

manufactured from a heterologous strain of PRRSV increased the SN antibody titer. An autogenous vaccine is one type of KV that is manufactured from a homologous strain of PRRSV. The autogenous vaccine is manufactured by collecting a specific strain of PRRSV that exists in a herd. Then, the virus specific strain is inactivated with an inactivating agent and formulated by using an adjuvant prior to administration. A subunit vaccine is manufactured by identifying the epitope that is responsible for inducing SN antibodies, then producing them by a recombinant DNA technology and, finally, formulating the expressed proteins with an adjuvant or by combining them with immunomodulators such as cytokines. These methods would or most likely produce an epitope responsible for SN antibody to the host.

Another notable observation in the present study is that pigs having SN antibodies were protected from subsequent rechallenge by another virulent heterologous strain, as observed by the absence of viremia. Only pigs in the negative control and KV-alone groups were viremic upon challenge. These observations suggest that SN antibodies could provide protection. In the present study, a challenge model was used in which pigs with SN antibodies were rechallenged intranasally with a high level of PRRSV. However, in field situations, virus transmission occurs by direct contact from other pigs that were infected previously. Therefore, further research should be conducted to test if pigs with SN antibody response can be infected following commingling with pigs exhibiting acute infection.

Previously, the role of SN antibody against PRRSV infection has been unclear regarding its effectiveness in protection. Perhaps it is more deleterious for the host due to its capability to enhance infection by antibody dependent enhancement (ADE) (Yoon et al., 1996). However, recent studies by Joo et al. (1999) and Osorio et al. (2001) indicate that PRRSV-specific antibodies, especially SN antibodies, are solidly correlated with protective immunity. Joo et al. (1999) did not find virus in any serum of sows that had SN antibodies following rechallenge. In contrast, virus was detected in sows that lacked SN antibodies. In that study, sows with SN antibody titers ranging from 4 to 64 were challenged with pooled PRRSV isolated from source herds, and the presence of virus was observed in serum after challenge. Recently, Osorio et al. (2001) passively transferred polyclonal antibodies that had neutralizing activity to non-immune sows. The sows were then challenged with PRRSV. Viremia was observed in sows and offsprings and vertical transmission from dams to offsprings. As evidenced by the complete absence of infectious virus in dams and their offspring, these

observations indicate that SN antibodies are associated with protection. Interestingly, polyclonal antibodies were employed in those studies.

Several monoclonal antibodies (Mabs) representing various antigenic determinants of PRRSV have been generated. In recent studies by Yang et al. (1999, 2000), three Mabs representing envelop- and matrix-associated proteins were shown to have neutralizing activity. It would be interesting to determine if these 3 Mabs can provide significant protection against infection. An experiment could be conducted by passively transferring those 3 Mabs to pigs to yield SN antibody titers, and then challenging the pigs with virus. The effect of specific Mabs on the magnitude and duration of viremia, and the duration of virus shedding will be evaluated. A shorter viremic response in the presence of Mab levels will indicate that antibodies to a specific antigenic determinant are primarily neutralizing in nature.

Attempts to eliminate and maintain herds free of PRRSV have been accomplished under certain conditions. First, signs of active virus circulation within a herd should no longer exist. The chance of virus transmission from pigs to pigs or from dams to their offsprings should also be reduced. Second, a herd's immunity should be more consistent. Since individual immunity within a herd exists at various levels, protocols to induce more consistent immunity should be considered to increase the level of herd protective immunity. An increase in the level of protective immunity would provide the pigs with resistance to reinfection to the herd. The current research determined the effect of KV treatment in pigs previously infected with PRRSV to enhance immune response in pigs previously infected with PRRSV. This protocol should be applied to a field study to maximize herd immunity against PRRSV infection and subsequently reduce a virus transmission. The parameters that could be used in the field study would be the reduction of virus transmission from dams to piglets. It would be interesting to determine if KV treatment can simultaneously increase herd immunity and reduce virus transmission from dams to piglets as determined by the PRRSV status of piglets.

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