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Duration of Infection and Proportion of Pigs Persistently Infected with Porcine Reproductive and Respiratory Syndrome Virus†

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Understanding the dynamics of porcine reproductive and respiratory syndrome virus (PRRSV) persistence in individual pigs is essential to the development of successful control programs. The objectives of this study were to investigate the proportion of inoculated pigs that become persistently infected with PRRSV and the duration of their infection. Additionally, different diagnostic techniques that detect persistent infections were compared. Twenty-eight 35-day-old pigs were inoculated with PRRSV. Serum and tonsil biopsy samples were collected on days 0, 7, 14, and 28 and then approximately monthly thereafter until day 251 postinoculation (p.i.). Tonsil, lymph node, and lung samples were collected following euthanasia on day 251 p.i. Virus was isolated from serum and tonsil biopsy samples that had been collected through days 28 and 56 p.i., respectively. Viral RNA was detected by reverse transcription (RT)-PCR in serum and tonsil biopsy samples that had been collected through day 251 p.i., although no serum samples collected from days 84 to 196 p.i. were positive and the presence of infectious PRRSV was not detected by swine bioassay of tissue samples collected at necropsy. The results confirmed that RT-PCR is more sensitive than virus isolation in identifying PRRSV-infected pigs. Six pigs that were persistently infected through days 225 or 251 p.i. remained seropositive throughout the study, although one pig had an enzyme-linked immunosorbent assay sample-to-positive ratio that was only slightly above the cutoff value of 0.40. Twenty of 28 tonsil biopsy samples collected on day 84 p.i. were positive by RT-PCR compared to only 1 positive biopsy sample out of 28 collected on day 119 p.i. The study's results indicate that most pigs clear PRRSV within 3 to 4 months, but that some may remain persistently infected for several months.

Porcine reproductive and respiratory syndrome virus (PRRSV) causes a potentially devastating disease in swine herds. Understanding the dynamics of PRRSV persistence and transmission is essential to the development of successful prevention programs. Research has documented PRRSV transmission between pigs in direct contact and allowed investigation of the duration of infection. Early transmission studies demonstrated that pigs were persistently infected and capable of transmitting virus 56 (8) and 99 (11) days after initial inoculation. The use of a convalescent, asymptomatic sow in the latter study illustrated the potential of recovered animals to be the source of infection to naïve herds. The offspring of PRRSV-inoculated sows were shown to transmit virus following treatment with immunosuppressive doses of prednisolone acetate at 154 days of age (1). Wills et al. provided direct evidence of persistent PRRSV infection in a study in which PRRSV was recovered from oropharyngeal scrapings up to 157 days postinoculation (p.i.) (9). Benfield et al. isolated virus from the tonsils of piglets up to 130 days after birth from sows inoculated at 85 to 90 days of gestation (4). In the same study, viral RNA was detected in the serum of one pig at 210 days after birth.

Field observations of closed herds infected with PRRSV for

long periods of time and instances of transmission via introduction of clinically normal, but PRRSV-infected, animals, into naïve herds highlight the importance of characterizing the persistence of PRRSV infection. The proportion of persistently infected animals also directly affects the dynamics of virus transmission within a herd. Since a persistently infected animal is a potential source of infection, the ability to estimate the proportion of persistently infected animals is of critical importance in developing strategies for prevention and control programs. Producers are often faced with the decision of whether to introduce previously infected animals into their herds. Currently, it is not clear if pigs that have returned to seronegative status following initial seroconversion are still capable of harboring PRRSV.

The objectives of this study were to investigate the proportion of inoculated pigs that become persistently infected with PRRSV and the duration of their infection. Additionally, this study compared different diagnostic techniques that allow detection of persistent infections.

MATERIALS AND METHODS

Virus. The PRRSV isolate (16244B) used in this experiment has been previously described (3). Briefly, the virus was originally derived from the serum of a 7-day-old clinically affected pig from a breeding herd that was experiencing a severe outbreak of porcine reproductive and respiratory syndrome. The virus was propagated twice in the MARC-145 cell line (7) at 37°C. The titer of the virus inoculum used in the study was determined by a microtiter assay of the same cell line.

Experimental animals. Fifty 10- to 14-day-old segregated, early weaned, female pigs were obtained from a herd that was periodically tested for PRRSV antibodies by enzyme-linked immunosorbent assay (ELISA) (HerdChek PRRSV; IDEXX Laboratories, Westbrook, Maine) and known to be free from

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PRRSV infection. The pigs were held in isolation rooms, acclimated to their surroundings for 17 days, and then randomly assigned to one of five isolation rooms according to treatment group. The pigs in four of the rooms were designated principals, while the pigs in the fifth group were designated negative controls. Five days later, when the pigs were approximately 35 days of age, serum samples were collected from the pigs. The pigs designated principals were inoculated intranasally with 2 ml of inoculum containing approximately 10^6 50% tissue culture infective doses of PRRSV isolate 16244B, divided between both nostrils. The negative controls were not inoculated with virus. Three pigs in each room were randomly assigned to another research study (2). They were treated the same as the other pigs in the rooms until 150 days p.i., at which time they were removed. Only information collected for the remaining 35 pigs is presented in this paper. The use of animals in this research project followed protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska—Lincoln and complied with all relevant federal guidelines.

Sample collection. Jugular or anterior vena cava blood samples were collected with blood collection tubes (Vacutainer; Becton Dickinson, Franklin Lakes, N.J.) on days 0, 7, 14, and 28 and then approximately monthly thereafter until day 225 p.i. After the blood samples were collected, the pigs were given an intravenous injection of an anesthetic cocktail that was made by reconstituting 250 mg of tiletamine and 250 mg of zolazepam (Telazol; Fort Dodge Animal Health, Fort Dodge, Iowa) with 12.5 ml of xylazine (100 mg/ml) and 12.5 ml of ketamine (100 mg/ml) (Ketaset; Fort Dodge Animal Health). The cocktail was administered at a dosage of 0.15 ml/10 lb of body weight. After the pigs were anesthetized, a 4.0-mm-diameter dermatology biopsy punch was used to harvest an approximately 4.0- by 8.0-mm section of tissue from each palatine tonsil. Immediately upon collection, one tonsil biopsy sample was placed in a sterile 1.5-ml Eppendorf tube on dry ice and stored at -70°C . The second biopsy sample, if collected on days 0 to 119 p.i., was placed in 0.5 ml of 10% buffered formalin. If collected on days 147 to 225 p.i., the second biopsy sample was placed on dry ice and stored at -70°C . On day 251 p.i., all inoculated pigs and one randomly selected control pig were euthanized. Blood, tonsil, bronchial lymph node, spleen, and lung samples were collected at necropsy. Portions of the tissue samples were placed in 10% buffered formalin. Other aliquots were frozen at -70°C . Blood samples were collected from the remaining six control pigs the next day (day 252 p.i.). Serum harvested from blood samples was aliquoted into 1.5-ml Eppendorf tubes. Serum samples were tested by using a commercial PRRSV ELISA (HerdChek PRRSV; IDEXX Laboratories). Serum was also stored at -70°C for later analysis.

Virus isolation. Virus isolation on MARC-145 cells was conducted with serum and tonsil biopsy samples collected from the principals and a negative control pig randomly selected for a given collection day. Virus isolation was conducted with serum collected on days 7 to 84 p.i. and with tonsil homogenates prepared from biopsy samples or necropsy tissues collected on days 7 to 251 p.i. In each case, samples collected on the same day were assayed as a group. Tonsil homogenates were made by grinding the tissue with a pestle in an Eppendorf tube, adding 500 μl of minimal essential medium (MEM) with 50 μg of gentamicin per ml and vortexing with the pestle in the tube. The pestle was removed, an additional 500 μl of MEM was added, and the mixture was vortexed. The suspension was then passed through a 0.20- μm -pore-size filter and divided into two aliquots. One aliquot was used for virus isolation. The other aliquot of each tonsil homogenate from days 7 to 251 p.i. was reserved for analysis by nested reverse transcription (RT)-PCR.

Monolayers of 2- to 3-day-old MARC-145 cells in 12- or 24-well plates were used in virus isolation. The cells were inoculated with either 300 μl of serum or 150 μl of tonsil homogenate plus 150 μl of MEM with 50 μg of gentamicin per ml and incubated for 1 h at 37°C with 5% CO_2 . The media were removed by pipette and then replaced with 1 ml of MEM with 50 μg of gentamicin per ml. The cells were incubated for 7 days at 37°C with 5% CO_2 and checked daily for signs of cytopathic effects (CPE). If CPE were observed or the 7 days had elapsed, the plates were frozen and thawed. Two hundred microliters of the supernatant was used to inoculate 2- to 3-day-old MARC-145 cells. The procedure for the first passage was duplicated. At the end of the 7-day incubation period or if CPE were seen sooner, the cells were fixed with acetone-methanol and stained with the PRRSV fluorescent monoclonal antibody conjugate SDOW17 (David Benfield, South Dakota State University, Brookings, S.Dak.).

RT-PCR. Aliquots of the tonsil homogenates prepared as inoculum for the virus isolation assay were analyzed by RT-PCR. Separate tonsil biopsy samples collected on days 147 to 225 p.i. and tonsillar tissue collected at necropsy were prepared specifically for RT-PCR analysis. In this case, the RNA extraction process was conducted directly on ground tonsillar tissue, not on tonsillar tissue mixed with MEM. The presence of viral RNA was also assessed by RT-PCR with serum samples collected on days 0 to 251. Samples that were collected on the

same day were assayed as a group, with the exception of the serum samples collected from control pigs on day 252 p.i., which were analyzed with the samples collected on day 251 p.i.

Viral RNA was extracted by first adding 0.75 ml of Trizol (Gibco BRL, Gaithersburg, Md.) to 0.25 ml of serum, 0.25 ml of inoculum prepared for virus isolation from tonsil tissues, or directly to ground tonsillar tissue. A known PRRSV-positive serum sample and a PRRSV-negative fetal calf serum sample were included as positive and negative controls. After incubation at room temperature for 5 min, 0.2 ml of chloroform was added to each sample, which were shaken vigorously for 15 s, incubated for 15 min at room temperature, and then centrifuged at $10,000 \times g$ for 15 min at 4°C . The supernatant was removed and added to tubes containing 2 μl of glycogen. After 0.5 ml of isopropanol was added, the contents of the tubes were mixed and incubated at room temperature for 10 min. Following centrifugation at $10,000 \times g$ for 10 min at 4°C , the isopropanol was drained, leaving a pellet that was then rinsed with 1 ml of 75% ethanol–25% diethyl pyrocarbonate water and allowed to dry. The RNA pellet was then dissolved in 20 μl of diethyl pyrocarbonate water. RT-PCR was performed by using an EZ rTth RNA PCR kit (Perkin-Elmer, Branchburg, N.J.) and was followed by nested PCR, both using primers which amplify open reading frame 6 of PRRSV. These primers included 14424 F (5' AGGTGCTCTTGGC GTTCTCTATT 3') and 14836 R (5' GCTTTTCTGCCACCAACACG 3') for outer PCR and 14661 F (5' CCTCCAGATGCCGTTTGTGCT 3') and 14790 R (5' TGCCGTTGACCGTAGTGGAGC 3') for nested PCR.

Each reaction was performed in a final volume of 50 μl containing 4 mM Mn^{2+} (for the RT), a 0.3 mM concentration of each deoxynucleoside triphosphate, a 0.8 μM concentration of each primer, and 5 U of a recombinant *Tth* polymerase. The samples were then placed in a thermocycler for 30 min at 60°C and then for 3 min at 95°C , followed by 35 cycles consisting of 1 min of denaturation at 95°C , 1 min of primer annealing at 60°C , and 1 min of extension at 72°C . For the nested PCR, 2- μl aliquots of products from the previous RT-PCR were added to tubes containing 1 mM Mg^{2+} , a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.8 μM concentration of each primer, and 5 U of *Taq* polymerase. Cycling parameters for the nested PCR were the same as those for the outer PCR. The samples were then electrophoresed on 2% Nu-Sieve 3:1 agarose (FMC BioProducts, Rockland, Maine).

Bioassay. The presence of infectious PRRSV in tissue samples collected at necropsy from the 28 principals and one control pig was assessed by bioassay. PRRSV-free sentinel pigs were inoculated with tissue preparations from the principals and the control pigs and then tested for evidence of infection. Samples of tonsil, lymph node, and lung tissue were used for pigs 1, 2, 16, 24, 26, 29, 34, and 40. The procedure was subsequently modified to also include samples of spleen to potentially improve the detection of virus in the remaining pigs. Approximately 8 g of each type of tissue was minced and mixed with 15 ml of MEM. Homogenates were placed in plastic bags and macerated (stomacher; Colworth), frozen at -70°C , thawed, and centrifuged at $1200 \times g$ for 10 min. Eleven milliliters of supernatant was drawn into a syringe for inoculation of pigs. Serum samples (0.5 ml) collected from pigs 7, 29, and 40 on days 56, 251, and 225 p.i., respectively, were also used but without further processing. Eight 2- to 3-week-old pigs, obtained from a herd known to be PRRSV-free through periodic serologic testing, were used as sentinels. The pigs were housed in separate isolation rooms for the remainder of the experiment. For each round of bioassays, supernatant or serum from a single pig was injected into the peritoneal cavity of a sentinel pig. Serum samples were collected from sentinel pigs just prior to injection and again approximately 14 and 28 days later. Serum samples were assayed by PRRSV ELISA for the presence of anti-PRRSV antibodies. The eight recipient pigs remained seronegative and were used repeatedly as sentinels for all subsequent bioassays.

RESULTS

Serum and tonsil biopsy samples collected from randomly selected control pigs were negative by virus isolation on all sample days tested. All tonsil samples and serum samples collected from negative-control pigs were RT-PCR negative. PRRSV was isolated from inoculated pigs up to day 28 p.i. from serum samples and up to day 56 p.i. from tonsil preparations (Table 1). Positive RT-PCR results were obtained with the serum samples from 27 of 28 pigs on day 7 p.i., 28 of 28 pigs on day 14 p.i., and then sporadically through day 251 p.i. (Table 1). For the majority of the inoculated pigs, aliquots of

TABLE 1. Detection of PRRSV from serum and tonsil tissue samples

| Day p.i. | Virus isolation ^a | | RT-PCR ^b | | |
|------------------|------------------------------|----------------------|---------------------|---------------------------------|----------------------|
| | Serum | Tonsil biopsy sample | Serum | Tonsil VI inoculum ^c | Tonsil biopsy sample |
| 7 | 28/28 | 26/28 | 27/28 | 27/28 | NT ^d |
| 14 | 20/28 | 23/28 | 28/28 | 28/28 | NT |
| 28 | 2/28 | 9/28 | 7/28 | 27/28 | NT |
| 56 | 0/28 | 4/28 | 1/28 | 21/28 | NT |
| 84 | 0/28 | 0/28 | 0/28 | 20/28 | NT |
| 119 | NT | 0/28 | 0/28 | 1/28 | NT |
| 147 | NT | 0/28 | 0/28 | 2/28 | 0/28 |
| 168 | NT | 0/28 | 0/28 | 0/28 | 1/28 |
| 196 | NT | 0/28 | 0/28 | 0/28 | 1/28 |
| 225 | NT | 0/28 | 1/28 | 1/28 | 1/28 |
| 251 ^e | NT | 0/28 | 1/28 | 2/28 | 0/28 |

^a Values are numbers of serum or tonsil biopsy samples from which PRRSV was isolated relative to numbers tested.

^b Values are numbers of serum or tonsil samples positive for PRRSV RNA relative to numbers tested by RT-PCR.

^c Tonsil VI inoculum, aliquot of tonsil preparation used for virus isolation.

^d NT, not tested.

^e Samples collected on day 251 p.i. were at necropsy.

tonsil preparations used for virus isolation were RT-PCR positive through day 84 p.i. and then sporadically through day 251 (Table 1). Tonsil biopsy samples prepared specifically for RT-PCR analysis were all negative on days 147 and 251, and 1 tonsil biopsy sample out of 28 was positive on days 168, 196, and 225 p.i. (Table 1).

Serum or tonsil samples from nine of the inoculated pigs were RT-PCR positive at least once on day 119 p.i. or later (Table 2). The detection of viral RNA by RT-PCR from days 119 to 251 p.i. was sporadic in that viral RNA was detected in samples from nine different animals during this time, but RNA was not detected in samples from the same pigs during consecutive months. Furthermore, viral RNA was not detected in both serum and tonsil samples collected on the same day from any of the pigs.

All principal and control pigs were seronegative (ELISA sample-to-positive [S/P] ratios of less than 0.40) on day 0 p.i. The negative-control pigs remained seronegative throughout the experiment. All inoculated animals seroconverted. Three pigs returned to seronegative status on or after day 196 p.i. Virus was not isolated nor was viral RNA detected in these animals beyond day 119 p.i. The nine pigs that were persistently infected for 119 days or more according to RT-PCR results did not return to seronegative status by the end of the trial, although the S/P ratio for pig 1 was 0.41 on day 251 p.i. (Fig. 1). The aliquot of the tonsil homogenate prepared for virus isolation from this pig was positive by virus isolation up to day 28 p.i. and by RT-PCR on days 28 and 225 p.i.

The bioassay pigs were seronegative prior to the initial injection and remained so after repeated intraperitoneal injections. These results indicated that there was no infectious virus in the sera or tested tissue samples from day 251 p.i.

DISCUSSION

There appears to be a growing body of evidence suggesting that changes affecting persistence occur around 3 or 4 months

after initial infection. In the present study, there was an abrupt drop in the proportion of pigs with RT-PCR-positive tonsil samples from 71% on day 84 to 4% on day 119 p.i. Other investigators have demonstrated that a high proportion (90%) of inoculated pigs remain infected with virus through day 105 p.i. at least (6). The results of the present study also corroborate earlier work in which PRRSV was isolated from oropharyngeal scrapings collected from three out of four pigs on day 84 p.i., but the virus was isolated from, at most, one out of four samples collected on days 98, 115, 128, 143, and 157 p.i. (9). Similarly, in a study which used same-room cohorts of the pigs in the present study, Allende et al. reported that 5 out of 10 inoculated pigs were persistently infected at 84 days p.i. and that 2 out of 10 were infected on day 150 p.i. (2). Collectively, the results demonstrate that although pigs can remain persistently infected for several months, there is a distinct drop in the proportion of animals harboring the virus that suggests that most pigs clear the virus between 3 and 4 months after infection. The study does not rule out—and, in fact, it may suggest—that very low levels of replication may occur in individual animals and may allow sporadic detection of viral RNA.

Recent research (6) has indicated that the diagnostic sensitivity of RT-PCR analysis of oropharyngeal scrapings is greater than that of tonsil homogenates, which were used in the present study. Consequently, some variation between reports in the proportions of pigs and the lengths of time that they harbor PRRSV is to be expected. Perhaps, if oropharyngeal scrapings instead of tonsil biopsy samples had been used in the present study, PRRSV would have been detected in a greater proportion of pigs for a longer period of time.

Greater proportions of serum and tonsil biopsy samples from days 28 and 56 were found to be PRRSV RNA positive by RT-PCR than by virus isolation. These results confirm that RT-PCR is more sensitive than virus isolation in identifying PRRSV-infected pigs. It should be noted that positive RT-PCR results do not necessarily indicate the presence of viable virus, only the presence of viral RNA. At the same time, it appears likely that in order for viral RNA to be detected up to day 251 p.i., replicating virus must also be present for extended periods of time. This was supported by concurrent research in which the bioassay of pigs demonstrated the presence of viable

TABLE 2. RT-PCR results for pigs in which viral RNA was detected on day 119 p.i. or later

| Pig | RT-PCR result ^a on indicated day p.i. | | | | | | | | | | |
|-----|--|------|------|----|----|-----|-----|-----|-----|-----|-----|
| | 7 | 14 | 28 | 56 | 84 | 119 | 147 | 168 | 196 | 225 | 251 |
| 1 | A, S | A, S | A | | | | | | | | A |
| 2 | A | A, S | A | A | A | | A | | | | |
| 16 | A, S | A, S | A | A | A | | A | | | | B |
| 24 | A, S | A, S | A, S | | A | | | | | | A |
| 25 | A, S | A, S | | A | A | | | B | B | | |
| 26 | A, S | A, S | A, S | A | A | | | | | | A |
| 29 | S | A, S | A | | A | | | | | | S |
| 34 | A, S | A, S | A | | A | A | | | | | |
| 40 | A, S | A, S | A | A | | | | | | | S |

^a A, viral RNA detected in inoculum of tonsil virus isolation assay by RT-PCR; S, viral RNA detected in serum sample by RT-PCR; B, viral RNA detected in tonsil sample by RT-PCR.

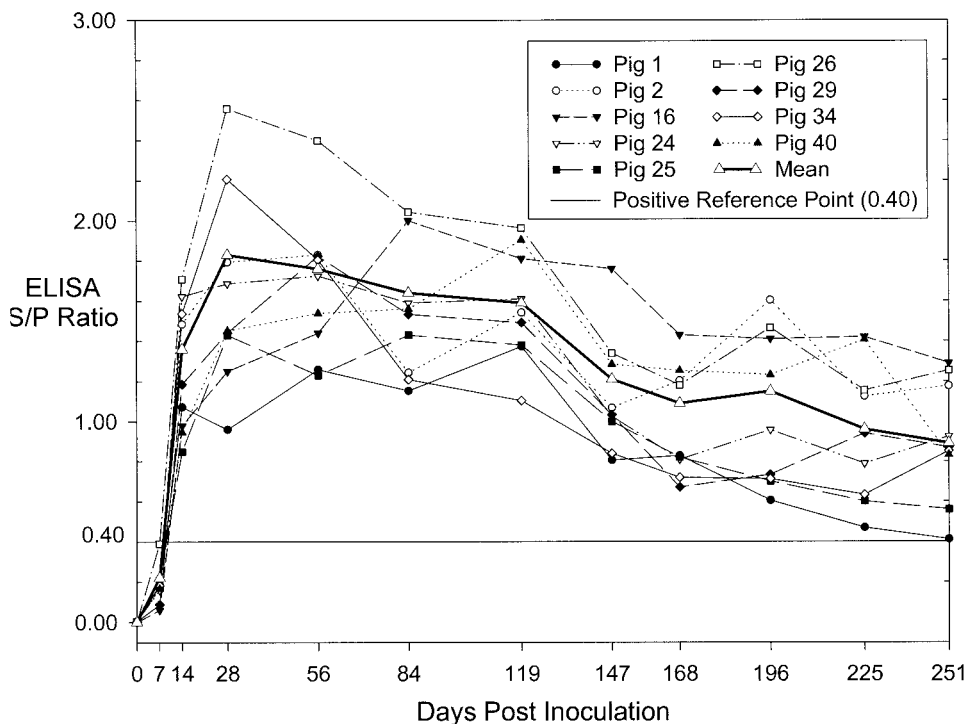


FIG. 1. PRRSV ELISA S/P ratios for persistently infected pigs over time.

virus in tissue samples collected from pigs inoculated 5 months previously (2).

PRRSV infection was cleared by day 251 p.i., as evidenced by negative bioassay results for tissue samples collected at necropsy. However, viral RNA was detected in tonsil samples collected from two pigs on day 251 p.i. Negative bioassay results were also obtained for three serum samples collected on days 56, 225, and 251 p.i. that tested positive for viral RNA by RT-PCR. The sporadic detection of positive results for viral RNA in serum after months of negative samples has been reported previously (4). Although it is possible that the observation of sporadic positive samples is due to false-positive reactions, 177 tonsil and serum samples collected from the seven negative-control pigs were negative by RT-PCR. If we assume that the RT-PCR results for the three serum samples and the two tonsil samples were truly positive, the apparent discrepancy between the RT-PCR and bioassay results would indicate that the bioassay is less sensitive than RT-PCR. In a study comparing bioassay and RT-PCR assay of boar semen samples, the bioassay detected the presence of virus in 63 of 67 RT-PCR-positive samples, reaffirming that RT-PCR may be more sensitive than bioassay (5). The infectious dose of PRRSV has been estimated to be less than 20 virions when inoculation is by the intranasal or intramuscular route (10). Consequently, bioassay would be expected to be an extremely sensitive test, but a greater infectious dose may be required when pigs are inoculated via the intraperitoneal route. Alternatively, the discrepancy could be explained by the persistence of viral RNA in the absence of viable virus. This is unlikely in that evidence supporting the persistence of RNA has not been previously reported.

With the exception of the data for pig 1, the results suggest

that an animal that has returned to seronegative status is unlikely to harbor the virus. The results for pig 1 suggest that viral RNA may still be detected in pigs with an S/P ratio of less than, or at least near, the cutoff value of 0.40. This finding has significant impact on the use of ELISA for the identification of PRRSV-infected animals. Pigs which have returned to seronegative status based on PRRSV ELISA results may still harbor PRRSV and be a source of virus for susceptible animals. A lower reference point might be indicated when the PRRSV ELISA is used for test and removal strategies. Additional research is needed to better determine the length of time during which pigs remain contagious to susceptible pigs.

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