

Using polymerase chain reaction to obtain PRRSV-free piglets from endemically infected herds

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Summary

Hysterectomy and early weaning (with onsite and offsite farrowing) were used in four endemically infected case herds to obtain piglets free from porcine reproductive and respiratory syndrome virus (PRRSV). Both conventional and one-step polymerase chain reaction (PCR) procedures were used to monitor the PRRSV status of the piglets.

Keywords: swine, porcine reproductive and respiratory syndrome virus (PRRSV), hysterectomy, early weaning, offsite farrowing, isolation, polymerase chain reaction

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Epidemiological studies have confirmed the presence of porcine reproductive and respiratory syndrome virus (PRRSV) in Canada since 1979 (Dea S, et al. *Proc Mystery Swine Dis. Comm Meet.* 1990; 67–72) and in the United States since 1985 (Joo HS, et al; *SIRS Comm Mtg.* LCI. 1992; 245–249. Owen WJ, et al; *SIRS Comm Mtg.* LCI. 1992; 243–244). Porcine reproductive and respiratory syndrome virus is now endemic in most pig producing countries. Once it has entered a herd, PRRSV tends to remain present and active in the herd indefinitely (Muirhead M, et al. *Managing pig health and the treatment of the disease.* 5M Enterprises Ltd. 1997;94–101,173–177). A number of management strategies have been used in the industry to try to control and/or eradicate PRRSV in herds.^{1,2} Early weaning³ and Isowean⁴—a modification of early weaning in which the sows farrow at the source farm—have been used to obtain weaned piglets free from certain infectious agents that are endemic in the herd.⁵ In this paper we describe three different management procedures:

- hysterectomy,
- early weaning with onsite farrowing, and
- early weaning with offsite farrowing

to obtain PRRSV-negative piglets from PRRSV-positive farms. We used a reverse transcription polymerase chain reaction (RT-PCR) to monitor the PRRSV status of the piglets.

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Polymerase chain reaction

Three primer sets were designed:

- a general primer set (P1/P2), which detects both American and European PRRSV isolates;
- an American-specific primer (P3), selected from a region near the 3' end of open reading frame (ORF) 7 and used with the upstream general primer (P1) in RT-PCR; and
- a European-specific primer (P4), selected near the 5' end of ORF7, and used with the downstream general primer (P2) in RT-PCR.

Degenerate positions were included in the sequences (Table 1) of the general primers to achieve 100% identity with the nucleotide sequence of 60% of the strains. With the remaining 40% of the strains, the identity was about 93%–97%, and the mismatch positions were never in the two nucleotides of the 3' end of the primer.

Ribonucleic acid was extracted from 100- μ L serum samples using a commercial reagent (Tripure Isolation Reagent, Boehringer Mannheim) following the manufacturer's protocol, based on the method previously described.⁶ The RNA pellet was then resuspended in 4 μ L of water.

Both conventional and a one-step RT-PCR were used to analyze samples with general primers. Specific primers were always used in conventional RT-PCR in this study. In conventional RT-PCR, reverse transcription of the viral RNA to obtain a cDNA was performed in a volume of 10 μ L that contained:

- 3 μ L of RNA sample,
- 1X PCR buffer II,
- 5 mM MgCl₂,
- 1 mM dNTPs,
- 1 μ M downstream primer,
- 10 U Rnase inhibitor, and
- 25 U reverse transcriptase (MuMIV).

Reaction was incubated 30 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 5°C. The amplification took place in 50 μ L of a reaction volume that contained:

- 10 μ L of the cDNA,
- 1X PCR buffer II,
- 2 mM MgCl₂,
- 0.2 mM dNTPs,
- 0.2 μ M both primers, and
- 1.25 U Taq polymerase.

Table 1

Primer sequences and their position on the Lelystad European and/or VR2332 American reference virus sequences.

Primer	Sequence (5' to 3')	Position genome
P1	CCAGCCAGTCAATCARCTGTG	14647 to 14667 (Lelystad) 2948 to 2968 (VR2332)
P2	GCGAATCAGGCGCACWGTATG	14938 to 14918 (Lelystad) 3248 to 3228 (VR2332)
P3	CATGCTGAGGGTGATGCTGTG	3271 to 3251 (VR2332)
P4	AGAAAAGTACAGCTCCGATGG	14619 to 14639 (Lelystad)

Nucleotide positions are numbered according to Lelystad⁸ and VR2332 sequence.⁹

The following protocol was used for amplification: 5 minutes at 94°C, 40 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and then 10 minutes at 72°C. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

For the one-step RT-PCR, AmpliTaq Gold (Perkin-Elmer Cetus Corp, Roche Molecular Systems, Inc.), an enzyme inactive at room temperature, was used instead of conventional thermostable DNA polymerase, and all the components of the RT-PCR reaction were mixed at the beginning of the reaction. Reactions took place in 25 µL of a reaction volume that contained:

- 2 µL of RNA sample,
- 1X PCR buffer II,
- 2.5 mM MgCl₂,
- 0.3 mM dNTPs,
- 10 U Rnase inhibitor,
- 6.25 U reverse transcriptase (MuMLV),
- 0.625 U of Taq Gold, and
- 0.4 µM of both primers.

Reverse transcription amplification was accomplished with the following protocol: 30 minutes at 48°C, 10 minutes at 95°C, 40 cycles at 95°C for 15s, and 60°C for 2 minutes and then 7 minutes at 72°C. Amplification products were analyzed by electrophoresis as in conventional RT-PCR. Because reverse transcription of the viral RNA and amplification of the cDNA was performed without manipulating the two reactions, the time it took to complete this assay was reduced. This allowed us to obtain results from the hundreds of samples involved in this study within 48 hours. It also reduced the possibility of errors and contamination.

The sensitivity of the conventional RT-PCR using the general primers was determined using tenfold serial dilutions of a viral suspension that had been previously titered (Spanish strain 5710, kindly supplied by Dr. E. Espuña, HIPRA Laboratories) in normal pig serum. Positive products were detected to dilutions containing 10^{1.6} TCID₅₀ per mL corresponding to RNA extracted from 3 TCID₅₀. The sensitivity of the one-step RT-PCR was the same as that of the conventional RT-PCR.

The specificity of the RT-PCR was determined by sequencing the amplified product obtained with the above virus suspension. PCR reac-

tions were then tested using various heterologous viruses (equine arteritis virus, a related arterivirus, classical swine fever virus, bovine viral diarrhea virus, swine influenza virus, rotavirus, parvovirus, African swine fever virus, and Aujeszky's disease [pseudorabies] virus) with titers between 10⁴ and 10⁶ TCID₅₀ per mL. This analysis was carried out with both general and specific American and European primers. The specificity of the isolates present in the positive samples were confirmed with positive batches typed using the specific American and European primers in the conventional PCR assay.

Case herds

Four herds endemically infected with PRRSV served as donor herds for all procedures used to obtain PRRSV- negative piglets in this study:

- Herd A was a 360-sow, farrow-to-finish herd in the United Kingdom,
- Herd B was a 420-sow, farrow-to-finish herd in the United Kingdom,
- Herd C was a 1000-sow, farrow-to-finish herd in the United Kingdom, and
- Herd D was a 100-sow, farrow-to-finish herd in Germany that farrowed every 3 weeks.

Clinical signs typical of PRRSV were not observed in any of these herds. None of the herds was being vaccinated against PRRSV.

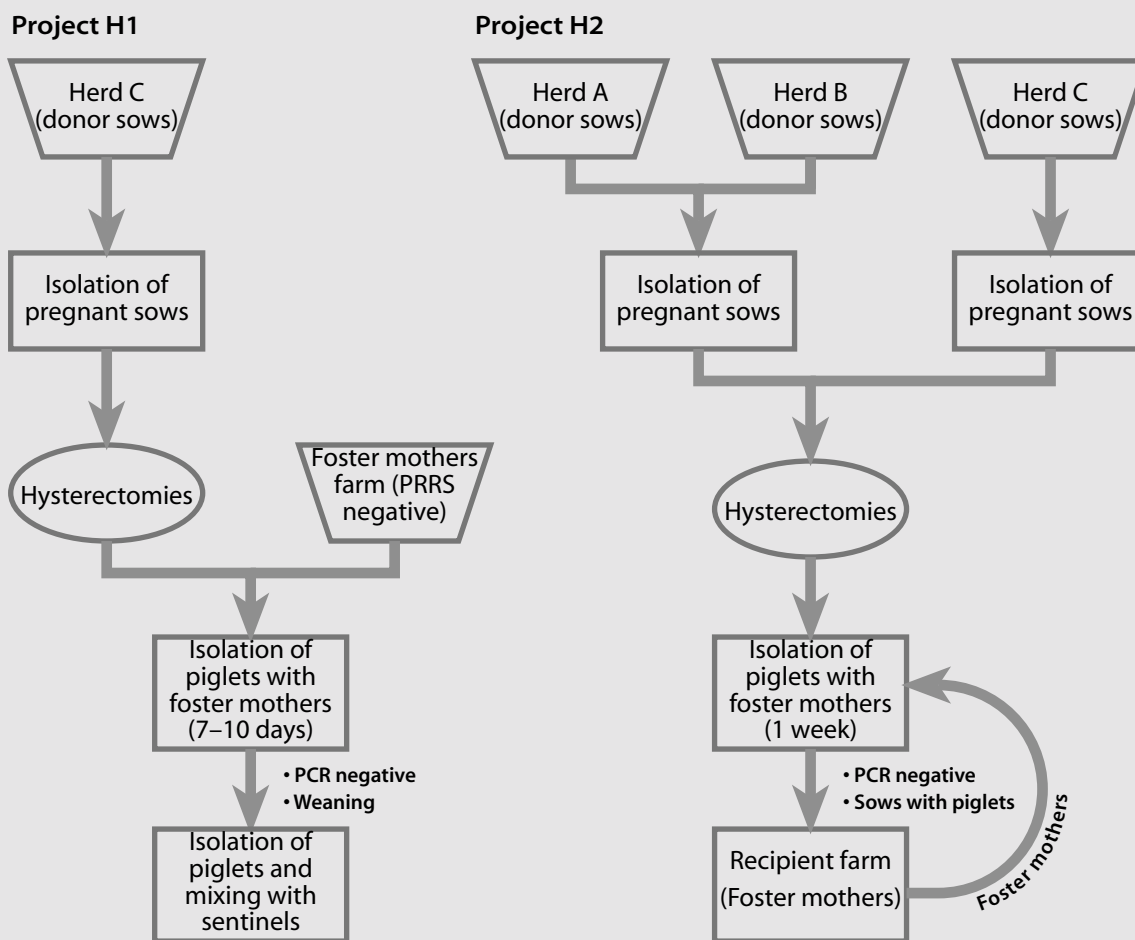
All procedures were conducted on batches of piglets; i.e., the number of piglets obtained by a given procedure at one given time. The procedure chosen at any point in a herd depended upon a combination of factors:

- the health status of the source farm (i.e., whether disease besides PRRSV, such as enzootic pneumonia, was present in the herd),
- the relative risks of transmitting disease, and
- cost.

Protocols

Hysterectomy

Two different hysterectomy projects (Projects H1 and H2) were conducted (Figure 1). In Project H1, donor sows from Herd C were moved into a continuous-flow isolation unit 30 days before farrowing. In Project H2, donor sows, at various times during gestation, were moved from Herds A and B to a continuous-flow isolation unit, and from Herd C, which had a different health status, to a different

Figure 1

Hysterectomy projects

continuous-flow isolation unit. In both hysterecctomy projects, sows were transported to a veterinary clinic, where hysterecctomies were performed using procedures standard for the clinic. All female piglets and one male piglet were selected from each litter for monitoring. Blood was collected from the umbilical cord of each piglet immediately after hysterecctomy and sent to the laboratory (Centro de Investigacion en Sanidad Animal [CISA], Spain) for PCR analysis.

Thirteen batches of piglets were obtained in Project H1 (i.e., all the piglets obtained from sows that underwent hysterecctomy on the same day), and 49 batches were obtained in Project H2. After hysterecctomy, piglets in both projects were fostered onto PRRSV-negative females and held in all-in–all-out (AIAO) isolation for 1 week.

If the serum of any piglet in a batch was found to be positive by PCR, then none of the piglets in that batch entered the recipient herd. In Project H1, all 13 batches were found to be PRRSV-negative by PCR, and so were weaned to another isolation facility on day 7 and placed in pens with PRRSV-negative age-matched sentinel pigs. Serosamples were collected from sentinel pigs on day 45 and antibodies to PRRSV were measured by ELISA. None of the sentinel pigs were found to have antibodies to PRRSV at day 45.

In Project H2, if PCR results were negative, the pigs and foster mothers were moved back on day 7 onto the foster mother's farm where study pigs were commingled with pigs in the foster recipient herd. Thirty random serum samples were taken from pigs in the recipient herd monthly to monitor for PRRSV by ELISA.

After the fourth batch of positive pigs was found in Project H2, we began to take blood samples on days 4–5 post-hysterecctomy, rather than on the day of hysterecctomy, to increase the chances of detecting positive piglets.

Of the 49 batches of pigs processed in Project H2, we detected five batches (39 piglets of 2013 piglets total in the second project, from 340 litters) being held in isolation as being positive to PRRSV by PCR (Table 2). Knowing which piglets were positive permitted us to keep piglets from positive batches out of the foster herd. The foster herd remained negative by serology for 10 months after the last batch of piglets was introduced from this study.

Early weaning projects

Three separate early-weaning projects were conducted to obtain PRRSV-negative piglets for three recipient herds. Donor herds

Table 2

Results of hysterectomy projects

Project	No. of batches	No. of hysterectomies	No. PCR	Source herd	Positive batches	No. of positive piglets
H1	13	123	805	Herd C	0	0
				Herd A	0	0
H2	49	217	2013	Herd B	Batches 27 and 29	Batch 27 (1 positive) Batches 29 (11 positive)
				Herd C	Batches 21, 24, 28, and 29*	Batch 21 (2 positive) Batch 24a (3 positive) Batch 28 (3 positive) Batch 29 (11 positive)*
Total	62	340	2818		5 Batches	

* Batch 29 PCR performed on serasamples on isolation day 4–5 post-hysterectomy (may explain increased number of positives)

participating in these early-weaning projects included Herds A, B, and D.

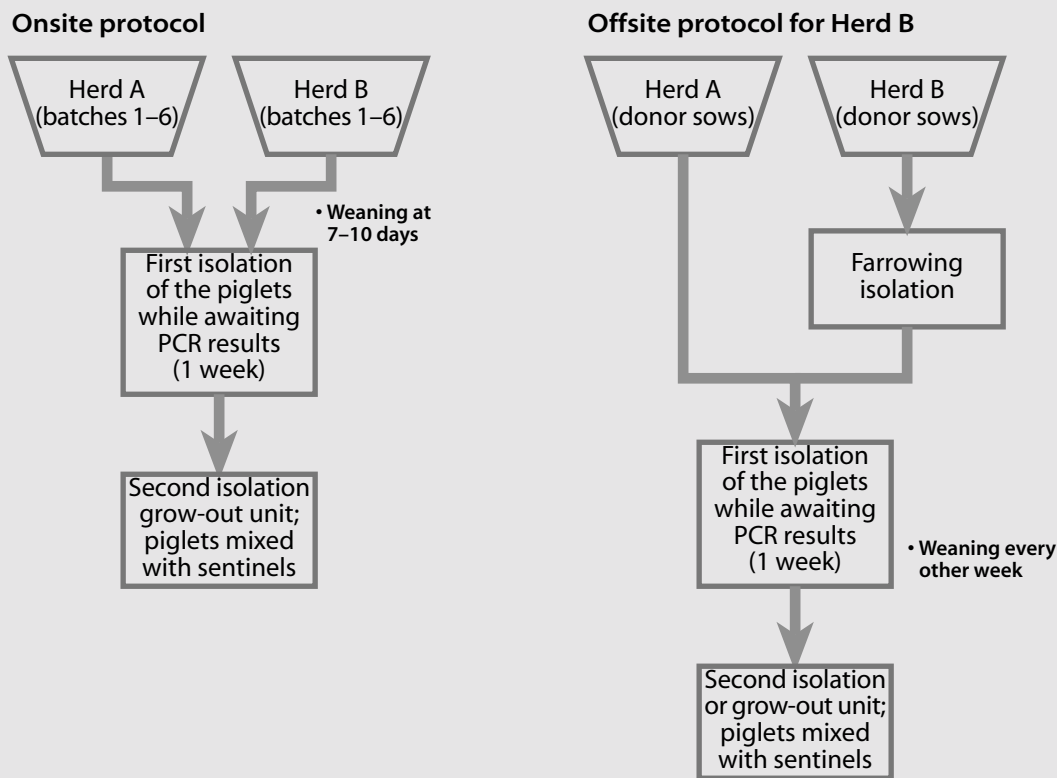
Project EW1

At the initiation of the first early-weaning project, sows from Herds A and B farrowed at the donor farm (Figure 2). Five female piglets and one male piglet were weaned every 2 weeks from each of these litters at 4–7 days of age into the offsite AIAO isolation unit. Blood samples were collected from piglets on the day of weaning and sent to CISA (Spain) for PCR analysis. If PCR results were negative, the piglets were moved to a second isolated grow-out unit at 11–14 days of age and

placed in pens with PRRSV-negative, age-matched sentinel piglets. Serosamples were collected from sentinel pigs on day 45 and samples were analyzed by ELISA.

Two batches of study pigs tested positive by PCR (Table 3). The two batches that contained positive piglets (three of 1452 piglets) were not placed in the second isolation unit. The first positive batch was euthanized. The second positive batch remained in isolation for another week while we performed virus isolation (VI), as previously described,⁷ to ensure that the results were not false positives. Sera were considered negative by VI if no PRRSV could be isolated after

Figure 2



Protocols for Project EW1, using onsite (left) and isolated offsite (right) farrowing

Table 3

Results of early weaning projects

Project	Average animals/batch	No. of PCR performed	Source farm	Onsite batches	Offsite batches	Positive batches	No. of positive piglets
EW1	121	1452	A B	9 6	0 6	Batch 4 (farm ID not available) and Batch 5 (from farm A)	Batch 4: 1 Batch 5: 2
EW2	95	668	D	7	0	none	0
EW3	105	1267	A B	12 0	0 12	Batch 1b Batch 2	1 1
Total	109	3387		34	18	On site: 3 batches Off site: 1 batch	

three “blind” passages. Cultures with cytopathic effect (CPE) were confirmed to be PRRSV positive by RT-PCR. Virus isolation results confirmed that this second batch was truly positive, so these pigs were euthanized.

After two batches tested positive and we obtained the sixth batch of piglets, we began taking sows from Herd B to an offsite isolation unit to farrow (Figure 2). An additional six batches of pigs were obtained by this modified offsite farrowing protocol, and three more batches were obtained with the initial onsite farrowing protocol from Herd A; none were positive for PRRSV by PCR (Table 3).

All sentinel piglets in the recipient isolation unit tested negative throughout the project.

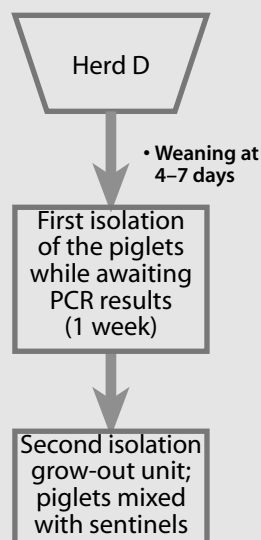
Project EW2

Simultaneously with Project EW1, we carried out Project EW2 in Herd D using the same onsite farrowing protocol we used at the beginning of Project EW1 (Figure 3). The seven batches we obtained from Herd D were all negative by PCR for PRRSV, and the sentinels all remained

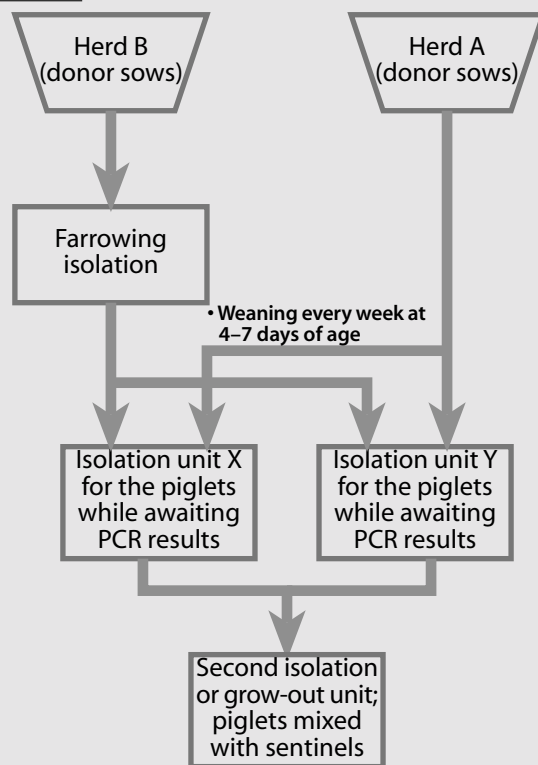
negative by ELISA for 45 days (Table 3).

Project EW3

Herds A and B were also used in this third early weaning project, conducted several months after the completion of Projects EW1 and EW2 (Figure 4). We used the same onsite farrowing protocol for Herd A and offsite farrowing protocol for Herd B (Figure 2) that we had used successfully in Project EW1 to monitor for PRRSV-positive piglets. In order to wean every week, pigs in Project EW3 were weaned alternately into one of two offsite isolation units, where they were held for 1 week postweaning while PCR was performed. Only pigs from negative

Figure 3

Protocols for Project EW2 using onsite farrowing

Figure 4

Protocols for Project EW3 using both onsite and offsite farrowing, weaning weekly into alternating isolation units

batches were moved into the second isolated grow-out unit.

One batch each from Herds A and B tested positive by PCR for PRRSV (Table 3), and these positive piglets/batches did not enter the grow-out facility. All sentinels in the recipient unit remained negative throughout this project.

Discussion

We were able to successfully obtain large numbers of PRRSV-negative piglets with all three of the management strategies we used in these projects, with

- 92% of the batches obtained by hysterectomy,
- 91% of those obtained with onsite farrowing/early weaning, and
- 94% of those obtained with offsite farrowing/early weaning testing PCR negative.

Although hysterectomy with fostering and early weaning (with either onsite or offsite farrowing) have been used prior to our study to produce PRRSV-negative piglets from PRRSV-positive farms, never before have these procedures been attempted on a large scale (Alexander TJLA; personal communication; 1998. Christianson WT, et al; *Proc. IPVS Congress*. 1994;68). Conventional PRRSV tests are too slow and inaccurate to provide the timely information needed for a large-scale study. The PCR provides a test sensitive and specific enough to detect minimal amounts of the virus in young piglets that have high concentrations of maternal antibodies, and can produce results within 48 hours of the arrival of the samples at the laboratory. The speed of the test allowed us to take the results into consideration within the necessary turnover time of the isolation units. The PRRSV PCR proved to be a useful and powerful technique that enabled the success of these projects.

Our choice of method to obtain PRRSV-negative piglets (either hysterectomy, early weaning with onsite farrowing, or early weaning with offsite farrowing) was motivated by the health status of the source farm, the risks involved in failing to detect positive piglets, and the costs. While it is a safer way to obtain piglets free of certain diseases, hysterectomies are expensive not only in terms of the cost of the surgical procedure but also because they require the loss of donor sows, use of foster mothers, and more specialized techniques to rear the piglets after birth and adapt them to farm conditions.⁵ Early weaning is a less expensive procedure, but can be difficult when young weaning ages are used.

The success of using hysterectomy to obtain PRRSV-negative piglets depends upon when the piglets are exposed to the virus. In an experimental infection of gilts/sows between 85–90 days of gestation, 77.5% of the pigs were born viremic (Benfield DA, et al. *Proc. AD Leman Swine Conf*. 1996:84–88). Because of the possibility of PRRSV transplacental infection, in our experience hysterectomy offered no additional benefits over early weaning (with either onsite or offsite farrowing) for producing PRRSV-negative piglets.

In the hysterectomy projects, piglets were tested immediately after hysterectomy for the practical reason of optimizing facility turnover.

However, testing the piglets after they had been in the isolation unit for 4–5 days, rather than immediately post-hysterectomy, resulted in us detecting more positive piglets in a positive batch (PRRSV was spreading quickly in the naïve piglets). Waiting a few days after hysterectomy to serosample seems a better system to ensure that one can detect positive piglets.

Our success in detecting positive piglets with all three of the strategies we used in these projects—i.e., hysterectomy plus isolated weaning, farrowing onsite plus isolated early weaning, or farrowing offsite plus isolated early weaning—suggests that isolating piglets until PCR testing is performed can successfully be used to detect any positive animal in time to prevent them from entering the recipient herd. Operating the isolation units for the newly obtained piglets in an AIAO manner was a key element for the final success of the projects.

Detecting PRRSV by PCR was sensitive enough to detect any positive animal in our projects. None of the batches that were introduced into the PRRSV-negative recipient farms or mixed with sentinels created any problems. The PCR was also sufficiently specific, as the batches that were detected as positive also had clinical signs and some of them were later confirmed to be positive by VI or further PCR tests. Serological confirmation was not always possible because of the presence of maternal antibodies.

In general the positive batches in both the hysterectomy projects and the early weaning projects were consecutive, suggesting that the virus was actively circulating among the breeding females during gestation, when transplacental infection could occur. Because not all the piglets in a litter were selected, we were not able to determine what percentage of animals in each litter were born or weaned with PRRSV. It seems that if the virus is not actively circulating in pregnant sows, and strict protocols are used, all three strategies can be successful. In the early weaning projects, it is also possible that the piglets did not receive enough colostrum, and maternal immunity was not adequate to protect them from PRRSV infection.

Both hysterectomies and early weaning (with either onsite or offsite farrowing) had a high rate of success in preventing PRRSV-positive piglets from entering the PRRSV-negative recipient herd.

Implications

- All the strategies we used—hysterectomies and fostering, isolated early weaning after onsite farrowing, or isolated early weaning after offsite isolated farrowing—were successful in identifying PRRSV-positive piglets before they could be introduced into a negative herd.
- The PCR was a useful and practical tool in rapidly assessing the PRRSV status of large numbers of piglets.
- The one-step PCR used in this study on some samples proved to be as sensitive and specific as the conventional PCR. In addition, it was completed more quickly.

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