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Detection of Porcine Reproductive and Respiratory Syndrome Virus in Boar Semen by PCR†

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Porcine reproductive and respiratory syndrome virus (PRRSV) causes a devastating disease in swine. The presence and transmission of PRRSV by boar semen has been demonstrated by using a swine bioassay. In this assay, 4- to 8-week-old pigs were inoculated intraperitoneally with semen from PRRSV-infected boars. Seroconversion of these piglets indicated the presence of PRRSV in semen. Seroconversion in gilts has also been demonstrated following artificial insemination with semen from PRRSV-infected boars. These methods of detecting PRRSV in boar semen are time-consuming, laborious, and expensive. The objective of this study was to develop a reliable and sensitive PCR assay to directly detect PRRSV in boar semen. Primers from open reading frames 1b and 7 of the PRRSV genome were used in nested PCRs. Virus was detected at concentrations as low as 10 infectious virions per ml in PRRSV-spiked semen. Specificity was confirmed by using a nested PCR and a ^{32}P-labeled oligonucleotide probe. The primers did not react with related arteriviruses or other swine viruses. The PCR assay showed good correlation with the swine bioassay, and both methods were superior to virus isolation. To consistently identify PRRSV in boar semen, the cell fraction was separated by centrifugation at 600 × *g* for 20 min, a lysis buffer without a reducing agent (2-mercaptoethanol) was used, and nondiluted and 1:20-diluted cell fractions were evaluated by PCR. PRRSV was not reliably detected in the seminal plasma fraction of boar semen.

Porcine reproductive and respiratory syndrome (PRRS) is now recognized as an important disease of swine throughout the world (1, 9). The disease affects pigs of all ages and causes poor conception rates, late-term abortions, and stillborn and weak pigs. Respiratory distress accompanied by fever, hyperpnea, lethargy, and high mortality rates is seen in suckling, weaned, and grow-finish pigs (14, 22). In 1991, a small RNA virus called the Lelystad virus (LV) was isolated in The Netherlands and was identified as the cause of PRRS (22). Subsequently, a similar virus (VR-2332) was identified and characterized in the United States (2, 5). Both the LV and the VR-2332 PRRS virus (PRRSV) isolates have morphologic, physicochemical, and genetic properties similar to those of the arteriviruses, which include equine arteritis virus, lactate dehydrogenase elevating virus, and simian hemorrhagic fever virus (2, 6, 12, 22).

While the etiologic agent of PRRS has been identified and characterized, many aspects of the disease have only recently been described. Investigations in the United Kingdom (15) and the United States (19, 24) indicated that infected boars transmitted PRRSV in semen. These reports have further heightened concerns in the world swine industry, which is becoming more reliant on artificial insemination and the use of boar semen to introduce new genetic information into high-health-status herds. There are also concerns that trade restrictions may be placed on semen from countries with PRRSV-positive pigs (19). Identification of viruses in semen by conventional methods such as virus isolation (VI) is difficult to perform because of the cytotoxicity of semen samples (18). A swine bioassay (19) has also been developed whereby 13 to 15 ml of semen is injected intraperitoneally into a 4- to 8-week-old pig. This pig is then monitored for seroconversion to the PRRSV. Both of these methods are time-consuming, laborious, and expensive. However, PCR has the potential to provide a sensitive, rapid, and specific method to detect PRRSV in boar semen. PCR has been previously utilized in identifying human immunodeficiency virus (11), bovine herpesvirus 1 (20, 23), and equine arteritis virus (3) in human, bovine, and equine semen, respectively.

In this study, we describe the development of PCR for detecting PRRSV RNA in boar semen. A comparison was made among PCR, the swine bioassay, and VI for their ability to identify PRRSV in boar semen. Good correlation between PCR and the swine bioassay was observed, but poor sensitivity was obtained when VI was used. Consistency in detecting PRRSV-infected semen was obtained when the cell fraction of the semen was used for PCR analysis. A lysis buffer without a reducing agent (2-mercaptoethanol) was utilized for RNA extraction, and an undiluted cell fraction and a 1:20 dilution of the cell fraction were also needed to obtain optimal results by PCR. A PCR for detection of PRRSV in boar semen will be useful as a diagnostic tool, since PRRV transmission through boar semen has been demonstrated (24). PCR will also be useful in defining the pathogenesis of PRRSV in reproductive disease.

MATERIALS AND METHODS

Animals, experimental inoculation, and protocol. Five PRRSV-seronegative boars were obtained and housed at Iowa State University as previously described

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(19). Four of these boars (boars 115, 117, 119, and 125) were inoculated with 2 ml of the ATCC VR-2402 PRRSV isolates per naris at a concentration of $10^{6.5}$ 50% tissue culture infective dose per ml. Semen was collected twice weekly for 8 weeks following experimental inoculation (19). Boar 121 was used as an uninoculated control and semen was collected once weekly from this boar as previously described (19). Sperm-rich and sperm-poor fractions were collected separately and stored at −80°C in 4- to 5-ml aliquots (19).

Viruses and cells. The ATCC VR-2402 PRRSV isolate was originally isolated on porcine alveolar macrophages and adapted to an African green monkey kidney continuous cell line (MA-104) as previously described (19). For VI, MARC-145 cells were utilized (10). These cells were derived by cloning from the MA-104 parent cell line and yielded a higher virus titer than MA-104 cells (10).

VI. Two milliliters of whole semen was freeze-thawed, mixed with 20 ml of Hanks balanced salt solution, and centrifuged at 40,000 × *g* for 1 h. The supernatant was discarded, and the pellet was resuspended and vortexed in 1 ml of minimum essential medium with 2% horse serum. Confluent monolayers of MARC-145 cells were inoculated with 200 μl of the suspension at 1:2 and 1:40 dilutions in minimum essential medium with 2% horse serum. After incubation for 2 h at 37°C, the supernatant was discarded, 1.5 ml of replacement medium (minimum essential medium with 2% horse serum) was added to each well, and the inoculated cell cultures were then incubated at 37°C for 40 to 48 h. The supernatants were discarded, and 1 ml of 80% acetone was added to each well for 15 min and then discarded. Plates were air dried, and 200 μl of fluorescein-conjugated anti-PRRSV monoclonal antibody (SDOW 17) (13) was added to each well and incubated 30 min at 37°C. Wells were then washed three times with phosphate-buffered saline (PBS) (pH 7.2) and evaluated by fluorescence microscopy. If detectable virus-specific fluorescence was present in any cells of the monolayer, the sample was considered positive for PRRSV by VI.

Swine bioassay. The swine bioassay for the detection of PRRSV in semen has been previously described (19). Briefly, one 4- to 8-week-old pig was inoculated intraperitoneally with a 13- to 15-ml sample of semen (equal volumes of sperm-rich and sperm-poor fractions) from a single collection. Pigs were then monitored serologically at weekly intervals by using the indirect fluorescent-antibody test. Two or more consecutive indirect fluorescent-antibody-positive results from weekly blood samples of the same pig were considered indicative of infective PRRSV in the semen.

Separation of seminal fractions. For all but two samples, 1 ml of sperm-rich and 1 ml of sperm-poor fractions (19) were used for PCR. For boars 115 and 117 at 3 days postinoculation (DPI), 3 ml of sperm-rich and 3 ml of sperm-poor fractions were used. Semen was then separated into cellular and seminal plasma fractions by centrifugation at 600 × *g* for 20 min (8). Seminal plasma was removed to approximately 1 in (2.54 cm) above the cell fraction layer, and the remaining seminal plasma was discarded. The cell fraction was washed once with PBS and stored at −70°C. Seminal plasma was also stored at −70°C.

Sample preparation and extraction of PRRSV RNA. Semen samples were processed similarly to the method described by Chomczynski and Sacchi (4), with minor modifications. When either whole semen or seminal plasma fractions were tested, 500 μl was added to an equal volume of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% Sarkosyl [*N*-lauryl sarcosine], 0.1 M 2-mercaptoethanol). When the cell fraction of the semen was used, 500 μl of lysis buffer without 2-mercaptoethanol was mixed with an equal volume of cells. Fifty microliters of this mixture was then added to an additional 450 μl of lysis buffer without 2-mercaptoethanol for a 1:20 dilution. Siliconized polypropylene tubes were used for all extraction procedures (USA/Scientific Plastics, Ocala, Fla.). Five hundred microliters of the lysates was then added to 250 μl of phenol and 250 μl of chloroform-isoamyl alcohol (24:1), and the mixtures were vortexed and centrifuged at 10,000 × *g* for 5 min. The upper aqueous phase was retained and extracted again with phenol-chloroform-isoamyl alcohol and once with 500 μl of chloroform-isoamyl alcohol. The volume of the upper phase was estimated, and 1/3 volume of 2 M sodium acetate (pH 4) and 2 volumes of cold 95% ethanol were added. The sample was chilled at −70°C for 1 h and then centrifuged for 30 min at 16,000 × *g*. Ethanol was carefully removed, and the pellet was washed twice with 100 μl of 70% ethanol. The pellet was then air dried and reconstituted in 30 μl of distilled water (Gibco, Grand Island, N.Y.).

PCR primers and probe. The primers and probe from open reading frame (ORF) 7 were designed in our laboratory and derived from the PRRSV VR-2332 sequence (a U.S. PRRSV isolate). Sequence information was kindly provided by Michael Murtaugh, University of Minnesota, St. Paul. The outer and nested primers were made by Integrated DNA Technologies, Inc., Coralville, Iowa. The outer sense and antisense primers were 5′-TCGTGTTGGGTGGCAGAAAA GC-3′ (nucleotides 2763 to 2785) and 5′-GCCATTCACCACACATTCTTCC-3′ (nucleotides 3247 to 3225), respectively. The nested sense and antisense primers were 5′-CCAGATGCTGGGTAAGATCATC-3′ (nucleotides 2885 to 2907) and 5′-CAGTGTAACTTATCCTCCCTGA-3′ (nucleotides 3121 to 3099), respectively. The internal probe was 5′-TGTCAGACATCACTTTACCC-3′ (nucleotides 3002 to 3022). Outer and nested primers derived from ORF 1b of the LV sequence (a European PRRSV isolate) were also used. The outer sense and antisense primers were 5′-CCGTCACCAGTGTGTCCAA-3′ (nucleotides 8751 to 8771) and 5′-CCGTTCTGAAACCCAGCAT-3′ (nucleotides 9003 to 8984), respectively. The seminested sense and antisense primers were 5′-ACATGG TATTGTCGGCCTT-3′ (nucleotides 8803 to 8822) and 5′-CGTTCTGAAAC CCAGCATC-3′ (nucleotides 9002 to 8983), respectively. For our study, only ORF 7 primers were utilized to evaluate semen samples by PCR, since the boars had been inoculated with the VR-2402 isolate (a U.S. PRRSV isolate).

Reverse transcription. The PRRSV RNA was reverse transcribed with reagents from a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, Calif.), and the reaction was carried out in oil-free tubes (Barnstead/Thermolyne, Dubuque, Iowa). Two microliters of extracted RNA was added to a mixture containing final concentrations of 5 mM $MgCl_2$ solution, 1× PCR buffer II (10× PCR buffer consisted of 500 mM KCl and 100 mM Tris-HCl), 1 mM each deoxynucleotide triphosphate (dNTP), 1 U of RNase inhibitor per μl, 2.5 U of reverse transcriptase per μl, and 0.75 μM antisense outer primer plus 1 μl of sterile distilled water for a 20-μl total volume per sample. All reagents and mixtures were kept on ice and then placed in a thermal cycler (Temp-tronic; Barnstead/Thermolyne) for 1 cycle at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min.

Outer and nested PCRs and visualization of PCR products. For outer DNA segment amplification, an 80-μl PCR mixture with final concentrations of 2 mM $MgCl_2$, 1× PCR buffer II, 0.15 μM outer sense primer, and 2.5 U of *Taq* DNA polymerase per 100 μl plus 65.5 μl of sterile distilled water was added to the same tube as the reverse transcription product. This mixture (100-μl total volume) was then placed in the thermal cycler for 40 cycles at 95°C for 25 s, 58°C for 5 s, and 74°C for 25 s. For nested DNA amplification, 2 μl of the outer DNA product was added to a fresh tube containing final concentrations of 3 mM $MgCl_2$, 0.4 mM each dNTP, 2.5 U of DNA *Taq* polymerase per 50 μl, and 2.4 μM each of the nested sense and nested antisense primers plus 8 μl of 10× PCR buffer II and 19.5 μl of sterile distilled water in a 50-μl total volume. The nested amplification was then performed in a thermal cycler for 30 cycles with the same annealing, denaturing, and DNA extension temperatures and times as those used for the outer reaction. After completion of the nested DNA amplification, 9 μl of each reaction mixture was mixed with 2 μl of a 1× sample buffer, type IV (17), and separated on a 1% SeaKem (FMC Bioproducts, Rockland, Maine) agarose gel containing 1 μg of ethidium bromide per 2 ml of agarose. A 100-bp DNA ladder (Gibco BRL, Grand Island, N.Y.) was used as a reference marker. The 484-bp outer ORF 7, 252-bp outer ORF 1b, 236-bp nested ORF 7, and 200-bp seminested ORF 1b products were then visualized and photographed under UV illumination.

Quality control for PCR. False-positive reactions may easily occur with PCR which involves the use of nested primers, since these primers amplify an already greatly amplified product. Precautions were taken to prevent these reactions. Four separate rooms were used for RNA extraction, outer and nested PCR, and agarose gel detection. In addition, lab coats and gloves were changed often, and aerosol-resistant pipette tips and dedicated pipetters were utilized for each step of the procedure. Positive and negative controls were also used with each group of eight PCR samples.

cDNA probe labeling and Southern hybridization. The internal oligonucleotide probe was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, Ohio). One microliter of 10× T4 polynucleotide kinase buffer (0.5 M Tris-HCl [pH 7.6], 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol) was added to 1 μl of 20 μM oligonucleotide probe. Seven microliters of 10-mCi/ml (3,000 Ci/mmol) [γ-^{32}P]ATP and 1 μl of T4 polynucleotide kinase (10 U/μl) were then added to the reaction tube. The solution was centrifuged briefly, mixed, and incubated for 10 min at 37°C. The reaction was then terminated by heating at 65°C for 5 min. For Southern blotting, the agarose gel was transferred to a nylon membrane with a rapid transfer system (Turboblotter; Schleicher & Schuell, Keene, N.H.). The DNA was fixed on the membrane with a UV crosslinker at 120,000 J/cm^2. The membrane was then placed on 6× SSC buffer for 2 min (20× SSC stock solution consisted of 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of water [pH 7], adjusted to 1 liter and autoclaved). Prehybridization was performed for 1 h at 42°C in 6× SSC buffer–0.5% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml–5× Denhardt's solution (50× Denhardt's solution consisted of 5 g of Ficoll, 5 g of polyvinylpyrrolidone, and 5 g of bovine serum albumin dissolved in water for a 500-ml total volume and filtered through a disposable filter) (16). The prehybridization solution was then discarded and the membrane was hybridized overnight at 42°C in 6× SSC buffer–10 mM EDTA–2 μl of labeled probe–0.5% SDS–100 μg of denatured salmon sperm DNA per ml–5× Denhardt's solution as previously described (16). The membrane was then washed twice for 5 min each time in 50 ml of a mixture of 2× SSC buffer and 0.5% SDS. The membrane was then air dried and exposed to an X-ray film (X-OMAT; Kodak) for 5 h.

RESULTS

Optimization of PCR amplification conditions. To improve the specificity and sensitivity of the PCR, various $MgCl_2$ concentrations (1.6 to 5 mM), primer concentrations (0.2 to 0.4 μM), annealing (50 to 58°C) and denaturing (94, 95, or 98°C) temperatures, and numbers of cycles (30, 40, or 45) were tested. The optimum product yield was achieved with 5 mM $MgCl_2$, 0.4 μM primer, annealing and denaturing temperatures of 58 and 95°C, respectively, and 30 cycles.

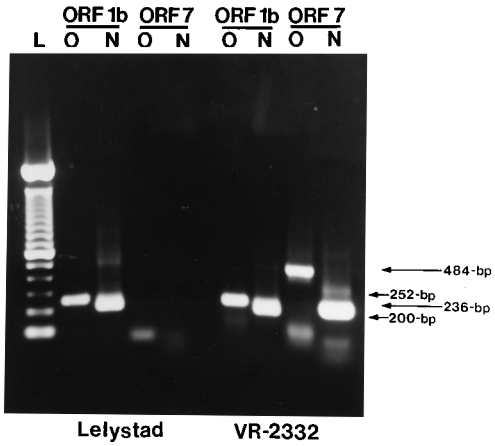

FIG. 1. Ethidium bromide-stained 1% agarose gel showing detection of the Lelystad and ATCC VR-2332 PRRSV isolates. Primers from ORF 1b (the polymerase gene) and ORF 7 (the nucleocapsid gene) are used for outer (O) and nested (N) reactions. A 100-bp DNA ladder (L) was used as a reference marker.

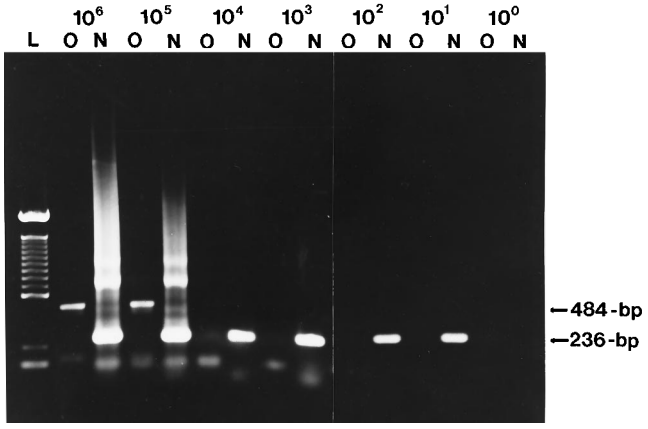

FIG. 3. Sensitivity of the PRRSV outer (O) and nested (N) PCRs. PCR products obtained from a 10-fold dilution series of viral DNA were analyzed on a 1% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (L) was used as a reference marker. The number of viral molecules detected at a 10^1 dilution was 10 virions per ml.

Specificity and sensitivity of the PCR amplification. Other arteriviruses (equine arteritis virus and lactate dehydrogenase elevating virus) did not react with primers derived from either ORF 1b or ORF 7 of the PRRSV genome. Primers from ORF 7 did not react with six other swine viruses (transmissible gastroenteritis virus, porcine respiratory coronavirus, rotavirus, pseudorabies virus, parvovirus, and swine influenza virus). To date, seven U.S. PRRSV field isolates obtained through the South Dakota State University Animal Disease Research and Diagnostic Laboratory have been tested with both ORF 1b and ORF 7 primers. All isolates reacted with both primer pairs. These primers have also been tested with the European PRRSV isolate LV. LV can be detected with primers designed from the polymerase gene (ORF 1b of LV) but not with primers derived from the nucleocapsid gene (ORF 7 of the U.S. VR-2332 isolate) (Fig. 1). The VR-232 PRRSV isolate can be detected with both primer pairs (Fig. 1). An internal radioactive probe was made from the ORF 7 VR-2332 sequence (Fig. 2).

The sensitivity of the PCR was also determined with a 10-fold dilution series of the VR-2332 isolate. As few as 10 virions (1 log unit of virus) per ml could be detected with the ORF 7 nested primers (Fig. 3).

Detection of PRRSV in boar semen by VI, the swine bioassay, and PCR. PRRSV from boar semen was infrequently identified by VI compared with the swine bioassay or PCR (Table 1). We did not observe significant cytotoxicity at a 1:40 dilution of semen, whereas cytotoxicity was occasionally observed at a 1:2 dilution of semen.

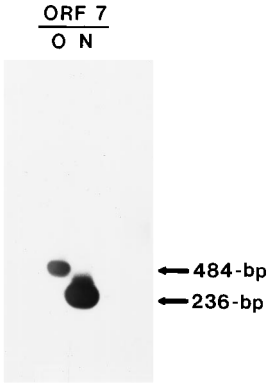

FIG. 2. Hybridization of the ^{32}P-labeled oligonucleotide probe to the outer (O) (484-bp) and nested (N) (236-bp) ORF 7 PCR products shown in Fig. 1.

PCR and swine bioassay results correlated except in 4 of 67 semen samples tested (Table 1). When performing PCR on boar semen, we often observed that some semen samples which were positive for PRRSV by the swine bioassay were negative by both VI and PCR. We theorized that there may be inhibitors in the seminal plasma or cell fraction of the semen that interfered with the PCR. To test this theory, we separated the cell fraction from the seminal plasma by centrifugation. Seminal plasma and cell fractions were then separately assayed by PCR. We observed good correlation between the PCR and swine bioassay results when the cell fraction was assayed by PCR. We did not detect PRRSV in the seminal plasma by PCR unless we concentrated the seminal plasma by high-speed centrifugation (40,000 $\times$ *g* for 1 h). However, only four of seven concentrated seminal plasma samples tested were positive (data not shown). Subsequent to this study, we inoculated an additional four boars with PRRSV and performed PCR on samples of their semen. Eight samples were tested, and all eight were negative when PCR was carried out on nonconcentrated seminal plasma fractions but positive when whole semen was used for PCR (data not shown). These results indicated that PRRSV is most consistently associated with the cell fraction of the semen but not with the seminal plasma.

DISCUSSION

The objective of this study was to develop a reliable, sensitive, and rapid test to directly identify PRRSV in boar semen. In our study, the PCR assay fulfilled these requirements. To consistently identify PRRSV-infected semen, we used low-speed centrifugation to separate seminal plasma from the cell fraction, extracted viral RNA from the cell fraction with a lysis buffer without a reducing agent on undiluted and 1:20-diluted cell fractions, and then carried out a nested PCR. The reliability of the PCR assay was supported by good correlation between the results of the swine bioassay and those of the PCR. Results from 63 of 67 semen samples tested showed correlation between PCR and the swine bioassay (Table 1). For the remaining four samples, the PCR assay was positive, while the swine bioassay was negative. This could have occurred for

TABLE 1. Detection of PRRSV in boar semen by various assays

Boar	Test[a]	Result on DPI[b]																										
		0	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47	49	51	53	56
115	Bio	−	+		+		+		+		+		+		+		+		+		−		+		−		−		−
	PCR	−	+*		+*		+		+		+*		+*		+*		+*		+*		+*		+		+*		−		−
	VI	−	−		−		+		−		−		−		−		−		−		−		−		−		−		−
117	Bio	−	+		+		+		+		+		+		+		−		−		−		−		−		−		−
	PCR	−	+*		+*		+		+		+		+*		+*		−		+*		−		−		−		−		−
	VI	−	−		−		+		−		−		−		−		−		−		−		−		−		−		−
119	Bio	−		+		+		+		−		−		−		−		−		−		−		−		−		−	
	PCR	−		+		+		+		−		−		−		−		−		−		−		−		−		−	
	VI	−		−		−		−		−		−		−		−		−		−		−		−		−		−	
125	Bio	−		+		+		−		+		+		+		−		−		−		−		−		−		−	
	PCR	−		+		+		+		+		+		+		−		−		−		−		−		−		−	
	VI	−		+		−		−		−		−		−		−		−		−		−		−		−		−	
121	Bio	−			−			−				−			−				−			−				−			−
	PCR	−			−			−				−			−				−			−				−			−
	VI	−			−			−				−			−				−			−				−			−

[a] Bio, swine bioassay.

[b] For the swine bioassay, + indicates seroconversion of a 4- to 8-week-old piglet inoculated intraperitoneally with PRRSV-infected boar semen. For PCR, +* indicates detection of a 236-bp nested PCR product resulting from the presence of PRRSV genomic RNA in the cell fraction of semen but not in whole semen and + indicates detection of a 236-bp nested PCR product resulting from the presence of PRRSV genomic RNA in whole semen. For VI, + indicates detection of PRRSV antigen following semen inoculation in MARC-145 cells, suggesting the presence of infectious PRRSV. −, no detection of PCR products, seroconversion of piglets, or detection of virus by VI.

several reasons. In the majority of samples from boars 115 and 117, the PRRSV RNA was detected only in the cell fraction and not in whole semen. This indicated that concentration of the semen was necessary for detection, and only a small amount of virus may have been present in the semen. Therefore, limited detection by the swine bioassay might occur. For boar 125, semen samples collected before and after 13 DPI were positive by both PCR and the swine bioassay, and this positivity occurred when viremia was present (19). Some authors have suggested that semen samples may be contaminated by serum or blood cells (21), and if viremia were present at 13 DPI, PRRSV might be found in the semen from serum or blood cell contamination. Therefore, the discrepancy was likely the result of a false-negative reaction involving the swine bioassay. Alternatively, since PCR detects physical particles (20) and not necessarily infectious virus, a positive PCR and negative swine bioassay result could indicate that infectious PRRSV was not present in that semen sample.

Increased sensitivity of PCR was observed when nested primers rather than outer primers were used as well as when the cell fraction of the semen was used. Initially, we centrifuged 1 ml of sperm-rich and 1 ml of sperm-poor semen fractions to obtain the cell fraction. This amount of semen was sufficient for correlating the results of the PCR with those of the swine bioassay for all samples except those from boars 115 and 117 (3 DPI). In these cases, we needed to use 3 ml of sperm-rich and 3 ml of sperm-poor fractions. Therefore, it appears advantageous to use a minimum of 6 ml of semen to obtain the cell fraction. Few virus particles may have been present on the first day of PRRSV shedding (3 DPI) for these two boars and a higher cell concentration may have been required for PRRSV to be detected.

Using the cell fraction, other authors have found an inhibition of the PCR due to sperm cell DNA (20, 23). To prevent this, we used a lysis buffer without 2-mercaptoethanol, a reducing agent similar to dithiothreitol. Dithiothreitol was used by Van Engelenburg et al. (20) and Mermin et al. (11) to obtain virus from sperm cells. Reducing agents appear to cause chromatin decondensation of sperm DNA (7) and this may cause false-negative PCR results. Another modification of PCR used to test semen was to dilute the cell fraction 1:20 in lysis buffer. Of the 30 cell fraction samples we tested, 5 (17%) were negative in the undiluted sample but positive in the diluted sample (data not shown). Therefore, the dilution allows detection of the virus in at least some of the samples in which virus might not be detected without dilution. The success of this procedure may result from the dilution of inhibitors present in the cell fraction.

PRRSV RNA extraction from the semen of PRRSV-positive boars has been previously described (21). These authors used low-speed centrifugation to pellet cells and then extracted RNA from the supernatant, and they concluded that there was no evidence for the possibility of transmission of PRRSV by semen. However, in our study, we consistently found PRRSV in the cell fraction and not in seminal plasma. Subsequent to our study, we inoculated an additional four boars with PRRSV and performed PCR on semen samples from these boars. To date, 15 seminal plasma samples have been tested by PCR and were negative for PRRSV, even though they were positive by the swine bioassay or by PCR on whole semen. Since 150 to 500 ml of boar semen may be obtained from a single collection, the PRRSV may not be present in large quantities or be evenly distributed in the semen. Therefore, a method of concentrating the sample and reducing interference from inhibitors in semen appears to be important for obtaining reliable PCR results.

Other extraction techniques and separations were attempted in order to obtain consistent results for PCR with boar semen. The extraction techniques described by Van Engelenburg et al. (20) and Chirnside and Spaan (3) and an RNaid (Bio 101, La Jolla, Calif.) procedure were used on the boar semen samples. We also concentrated whole semen samples and used seminal plasma samples (concentrated and nonconcentrated) to obtain

viral RNA from boar semen. However, these techniques were not consistent in identifying PRRSV-infected semen. When we obtained the cell fraction of boar semen by low-speed centrifugation and then used a lysis buffer without a reducing agent (2-mercaptoethanol) on the cell fraction for viral RNA extraction, PCR results consistently correlated with those of the swine bioassay. Both an undiluted cell fraction and a 1:20 dilution of the cell fraction in lysis buffer were used for PCR analysis.

Since we have primers which react with both U.S. and European isolates of the PRRSV, this assay will be useful as a diagnostic tool and will detect PRRSV in clinically ill and convalescent boars. The PCR assay will be particularly useful for semen supply companies that are under pressure to sell virus-free semen which could be used worldwide. It will also be valuable in determining the pathogenesis of PRRSV in reproductive disease. Further studies are needed to determine the effect of using PRRSV-infected semen to inseminate sows and/or gilts. Preliminary data has shown that seroconversion and decreased conception rates in gilts can occur from using this semen (24). Also, dissemination of the virus to other seronegative pigs could possibly occur from these sows and/or gilts. Since a vaccine for PRRSV infection in adult swine is not currently available, screening of semen samples for PRRSV is an important aspect of controlling this disease. In this study, we determined that PRRSV is predominantly found in the cell fraction of semen. Future studies are now in progress to determine if the virus is adsorbed to the spermatozoa or is actually carried in nonsperm cells or sperm.

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