RESEARCHREPORT
SWINE HEALTH

Title: Real-time PCR detection of PRRSv and rapid identification of vaccine in serum and semen **NPB #03-052**

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Abstract: The challenge studies completed in this study highlights the need for improving our Porcine Reproductive and Respiratory Syndrome virus (PRRSv) diagnostic capabilities. Correlating the current PRRSv infectious status of a boar using only one diagnostic approach from only one type of sample can result in misdiagnoses. The intent of this study was to evaluate the efficiency of a SYBR green "real-time" PCR for detecting PRRSv in boar semen and serum. When SYBR green PCR was compared to other PCR detection methods the SYBR green PCR was unable to detect the presence of PRRSv RNA in all serum and semen samples evaluated. While traditional electrophoretic gels stained with ethidium bromide (EtBr) did result in positive results and were superior to the SYBR green PCR results, Southern blot hybridizations with an internal DNA probe in hybridizations with the EtBr-stained PCR amplicons we were able to detect PRRSv RNA in samples that were otherwise undetectable by SYBR green or EtBr fluorescence detection. This highlights the need for a DNA sequence detection step in any PCR assay for detecting PRRSv RNA in semen based on these results and the potential for false-negative misdiagnoses. To overcome this, we recommend the development and evaluation of PCR approaches that incorporate automated DNA hybridization steps into the PCR analysis. Examples would be TaqMan or Molecular Beacon detection systems. These fluorgenic-based PCR assays would allow for improvement in "real-time" detection sensitivity and specificity that is lacking with the SYBR green detection system.

Also recognized in this study was the need for effective RNA recovery procedures from semen samples and their importance in the development of a sensitive PRRSv PCR detection procedure. Additional research will be required to identify and optimize the most efficient PRRSv RNA recovery processes from semen that ultimately meets the needs and requirements of the pork producer. The goal of the process should be the development of a straight-forward, user-friendly RNA recovery process that can be totally automated and be a component in a self-contained PRRSv-RNA detection system for on-farm applications.

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Introduction: Porcine Reproductive and Respiratory Syndrome virus (PRRSv) was initially described as a cause of reproductive and respiratory disease in the United States in 1987 and in Europe in 1991 and remains the most economically important swine disease in the world. The use of PCR-based assays for the detection of virus has aided our ability to identify infected pigs, especially those that are seronegative. PCR is also useful for the identification of virus shed in semen used for AI.

Even though PCR-based tests are relatively rapid and sensitive, they still require the submission of a sample to a diagnostic lab and analysis of results by diagnostic personnel. This processes is relatively time-consuming when using PCR to screen semen used for artificial insemination; a time-critical process where the producer would prefer an analysis of semen performed within minutes of collecting the sample. It is therefore obvious that there is a need for developing a polymerase chain reaction (PCR)-based diagnostic system that can perform rapid diagnosis of PRRSv infection, on site and by a practitioner.

Recent technology, developed in response to bioterrorism, have led to real-time (*rt*)-PCR systems that can detect infectious agents on site and deliver a positive or negative result within minutes of collecting the sample. Pre- and post-harvest food research groups at Kansas State University are actively involved in adapting this same technology for the rapid, real time analysis of viral and bacterial agents in animal samples. One emerging technology is the use of *rt*-PCR performed on a SmartCycler, a portable system manufactured by Cepheid (http://www.cepheid.com). When incorporated into the GeneXpert platform this technology will provide a self-contained automated system for performing all PCR-related steps, including sample preparation, reverse-transcriptase (RT)-PCR amplification, and the detection/interpretation of PCR, all within a single portable self-contained unit.

The current diagnostic laboratory-based *rt*-PCR assays for the detection of PRRSv in serum incorporate the use of primer pairs for the amplification of PRRSV sequence. The DNA products are detected with DNA sequence-specific probes, such as TaqMan or Molecular Beacons. Even though these probes are highly specific and target highly conserved regions of the virus, such as ORF7, there are concerns that small nucleotide sequence differences, which normally exist between isolates, could lead to the ability of some PRRSv field isolates to escape detection. Another approach for the detection of PCR products is the use of SYBR green, which fluoresces after binding to the doubled-stranded DNA PCR product and therefore the detection of the PCR product is not dependent on specific DNA sequence. Even though this approach is better at detecting all PRRSv isolates, regardless of sequence differences, it suffers from two shortcomings: 1) uncertainty about sensitivity, 2) lack of specificity, i.e. it detects only the presence of DNA, regardless of the source. These short-coming have been largely overcome by the SmartCycler, which can distinguish specific from non-specific PCR products based on a sophisticated analysis of the melting temperature (T_m) of the PCR products. The purpose of this proposed research project is the development of a sensitive and specific assay for the rapid detection of PRRSv in serum and semen, which can be performed using the SmartCycler system. With the integration of new sample handling and processing systems, such as the GeneXpert from Cepheid, this system will be able to detect PRRSv on site within 30 minutes of collecting the sample and with the same degree of accuracy and sensitivity as current diagnostic lab PCR assays.

Objectives: The objective of this project is to compare the sensitivity and specificity of SYBR green PCR with standard diagnostic techniques for the detection of PRRSv in boar semen and serum. The question this objective addresses is “can SYBR green

detection of PCR products generated using *rt*-PCR provide the same results as current PCR-based diagnostic tests?" The experimental approach is to determine the sensitivity and specificity of the SYBR green *rt*-PCR technique for the detection of PRRSv ORF7 in serum and semen in experimentally infected boars. Validity of the assay will be determined by comparing results of SYBR green *rt*-PCR to standard assays, including virus isolation (VI), RT-PCR and Southern blot DNA hybridization assays.

Materials & Methods:

a. Infection of animals, collection and storage of samples. Four boars from a PRRSv seronegative herd were infected intranasally with PRRSv lab isolate 23983 P6. Two additional boars were mock infected and served as negative controls. Blood and semen were collected weekly from each boar and divided into two equal aliquots; the first was retained and used for RNA extraction and immediate SYBR green RT-PCR amplification, the second aliquot was frozen at -80°C and saved fro later analysis of PRRSv by virus isolation (VI), PRRSv-antibody (serum only) using a commercial ELISA kit., and a diagnostic lab RT-PCR. (*rt*-PCR was performed immediately on all fresh serum and semen samples). Semen was collected and centrifuged to recover the cell fraction as previously described.[i] The cell fraction was retained and used for analysis of PRRSV.

b. SYBR green-based *rt*-PCR. RNA was isolated from 1 ml of serum or the cell fraction from 1 ml of semen using the Qiagen RNAeasy kit. Reverse transcription-PCRs on serum and semen samples were completed using PCR primers P71/P72 as previously described.[i] SYBR green *rt*-PCR was a modification of the current RT-PCR protocol used in the diagnostic lab at Kansas State University, and previously described,[i] except that SYBR green was incorporated into the PCR reaction and amplification performed using *rt*-PCR in a SmartCycler (Cepheid). At the end of the PCR run a melting curve analysis was performed to distinguish legitimate ORF7 gene products from non-specific products. Positive and negative controls for each assay include serum and semen from known PRRSV-positive and PRRSV-negative samples, respectively. Aliquots of each PCR were also loaded onto an agarose gel for electrophoretic resolution and visualized by staining with ethidium bromide (EtBr), then viewed under UV light. For additional detection sensitivity and specificity a Southern blot hybridization was preformed on the resulting SYBR green *rt*-PCR products using a digoxigenin-labeled oligonucleotide probe to the ORF7 PCR amplicon (5'-AGTGAGCGGCAATTGTGTCTG-3'). Hybridizations were completed using the Boehringer Mannheim DIG Nonradioactive Nucleic Acid Labeling and Detection System.

c. Virus isolation and serology. PRRSv virus isolation (VI) from serum and semen was performed on Marc 145 (Monkey kidney) cells using a standard protocol. PRRSv-specific antibody was measured using the IDEXX HerdCheck ELISA.

d. Analysis of data. Using this experimental approach we should be able to determine the accuracy and sensitivity of the SYBR green-based assay as it compares to current techniques. Accuracy of the SYBR green PCR method is determined by the agreement between SYBR green RT-PCR and results from the other assays. A lack of agreement will be in the form of false positives (diagnostic lab PCR and biological assays for PRRSV-infected pigs are negative for PRRSv because infected pigs are not shedding virus in serum or semen, however the SYBR green RT-PCR produces a positive result).Another type of false positive is what we call a "true" false positive. These results are positive for the detection of a SYBR green PCR ORF7 product in samples from the PRRSv-negative control group of pigs. The most likely source for

false positives is the inability to distinguish melting curves produced by ORF7 products from melting curves produced by nonspecific PCR products.

False negatives arise from infected pigs that are positive for PRRSv by diagnostic lab PCR and negative in the SYBR green assay. The most likely source of a false-negative result is the inability of SYBR green to detect small amounts of ORF7 DNA. We are most interested in knowing the number of "true " false-positives and false negatives, which has obvious implications for screening samples from seronegative herds.

Results:

a. Recovering PRRSV RNA from boar semen. A protocol previously described to detect PRRSv RNA from boar semen (Procedure #1) was evaluated.[i] This procedure involved using the Qiagen' Viral RNA kit directly on boar semen and evaluated by RT-PCR using the P71/72 primers.

Researchers at South Dakota State University have developed a proprietary procedure (Procedure #2) that uses a chemical method to solubilize the semen prior to using a commercially available RNA extraction kit (Qiagen RNeasy).[ii,iii]

In an effort to improve RNA recovery, we were interested in evaluating a procedure that utilizes bromelain[iv] to overcome PCR inhibition of semen prior to using the Qiagen Viral RNA Mini Spin kit (Procedure #3).

As a first step in the evaluation of these 3 different RNA recovery procedures serial-dilutions of PRRSv strain 23983 P140 were added to uninfected boar semen. Specifically, 100µl of tissue culture derived virus (estimated to have ~10^4 $TCID_{50}$/ml) was added to 900µl of PRRSv-negative boar semen and 10-fold serial dilutions were made using negative boar semen as the diluent. Each RNA procedure was then evaluated from each dilution and the RNA was then subjected to PRRSv-specific RT-PCR.

The detection limits for each RNA recovery process using RT-PCR primers to the ORF 7 region indicated that in our hands the RNA recovery procedures #1 & #3 were capable of detecting PRRSv when >10^1 $TCID_{50}$/ml or >$10^{-2 \text{ to } -3}$ $TCID_{50}$/PCR were present in boar semen (Table 1 & Figure 2). This contrasted with the detection threshold of procedure #2 with the same PCR primers in which PRRSv RNA was indicated when >10^2 to 10^4 $TCID_{50}$/ml or >$10^{0 \text{ to } -2}$ $TCID_{50}$/PCR were present in boar semen as identified in 3 different repetitions (Table 1 & Figure 2).

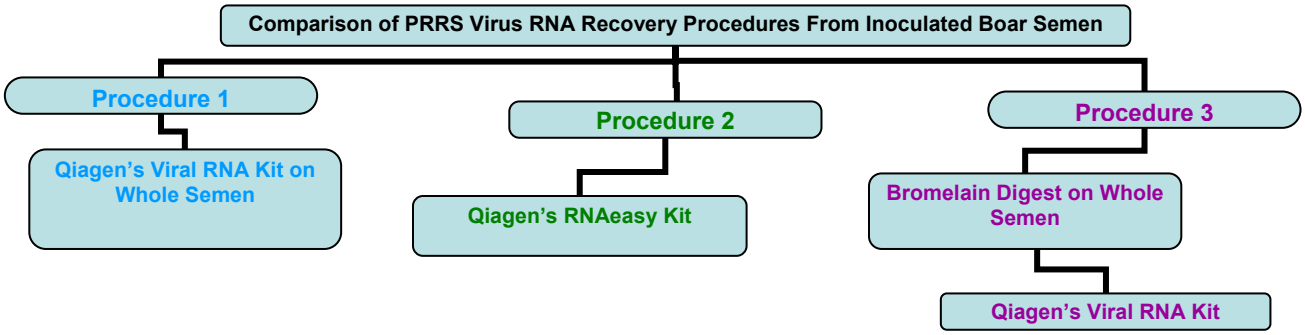

Figure 1. The above flow chart depicts the major steps involved in the recovery of PRRSV RNA that was inoculated into uninfected boar semen. Procedure #2 had more steps and manipulations, and did not result in the same level of detection of PRRSv RNA in inoculated semen samples as either Procedure #1 or #3.

Table 1. Detection limits of 3 different RNA recovery procedures to recover PRRSv RNA from PRRSv-inoculated boar semen. Efficiency of each recovery procedure was evaluated using 10-fold dilutions of tissue culture PRRSv that was inoculated into uninfected boar semen. The RNA was then subjected to PRRSv-specific RT-PCR[1] and interpreted by electrophoresis and visual resolution of the predicted PCR product in EtBr-stained agarose gels (See Figure 2). *Denotes actual virus concentration in each dilution. Positive PCR interpretations are indicated as PCR+ and are red; negative PCR interpretations are indicated as PCR- and are black. (Note: See Figure 2 for actual gels.)

Results of rep #1

RNA Recovery Procedure	*Dilution 10^{-1} PCR interpretation*	*10^{-2}*	*10^{-3}*	*10^{-4}*	*10^{-5}*	*10^{-6}*	*10^{-7}*	*10^{-8}*	*10^{-9}*	*10^{-10}*
#1	***$4e^{1}$ PCR+**	**4 PCR+**	**$4e^{-1}$ PCR+**	**$4e^{-2}$ PCR+**	$4e^{-3}$ PCR-	$4e^{-4}$ PCR-	$4e^{-5}$ PCR-	$4e^{-6}$ PCR-	$4e^{-7}$ PCR-	$4e^{-8}$ PCR-
#2	**$1e^{1}$ PCR+**	**1 PCR+**	**$1e^{-1}$ PCR+**	**$1e^{-2}$ PCR+**	$1e^{-3}$ PCR-	$1e^{-4}$ PCR-	$1e^{-5}$ PCR-	$1e^{-6}$ PCR-	$1e^{-7}$ PCR-	$1e^{-8}$ PCR-
#3	**$2e^{1}$ PCR+**	**2 PCR+**	**$2e^{-1}$ PCR+**	**$2e^{-2}$ PCR+**	**$2e^{-3}$ PCR+**	$2e^{-4}$ PCR-	$2e^{-5}$ PCR-	$2e^{-6}$ PCR-	$2e^{-7}$ PCR-	$2e^{-8}$ PCR-

Results of rep #2

RNA Recovery Procedure	*Dilution 10^{-1} PCR interpretation*	*10^{-2}*	*10^{-3}*	*10^{-4}*	*10^{-5}*	*10^{-6}*	*10^{-7}*	*10^{-8}*	*10^{-9}*	*10^{-10}*
#1	***$4e^{1}$ PCR+**	**4.0 PCR+**	**$4e^{-1}$ PCR+**	**$4e^{-2}$ PCR+**	$4e^{-3}$ PCR-	$4e^{-4}$ PCR-	$4e^{-5}$ PCR-	$4e^{-6}$ PCR-	$4e^{-7}$ PCR-	$4e^{-8}$ PCR-
#2	**$1e^{1}$ PCR+**	**1 PCR+**	$1e^{-1}$ PCR-	$1e^{-2}$ PCR-	$1e^{-3}$ PCR-	$1e^{-4}$ PCR-	$1e^{-5}$ PCR-	$1e^{-6}$ PCR-	$1e^{-7}$ PCR-	$1e^{-8}$ PCR-
#3	**$2e^{1}$ PCR+**	**2 PCR+**	**$2e^{-1}$ PCR+**	**$2e^{-2}$ PCR+**	$2e^{-3}$ PCR-	$2e^{-4}$ PCR-	$2e^{-5}$ PCR-	$2e^{-6}$ PCR-	$2e^{-7}$ PCR-	$2e^{-8}$ PCR-

Results of rep #3

RNA Recovery Procedure	*Dilution 10^{-1} PCR interpretation*	*10^{-2}*	*10^{-3}*	*10^{-4}*	*10^{-5}*	*10^{-6}*	*10^{-7}*	*10^{-8}*	*10^{-9}*	*10^{-10}*
#1	***$4e^{1}$ PCR+**	**4 PCR+**	**$4e^{-1}$ PCR+**	**$4e^{-2}$ PCR+**	**$4e^{-3}$ PCR+**	$4e^{-4}$ PCR-	$4e^{-5}$ PCR-	$4e^{-6}$ PCR–	$4e^{-7}$ PCR-	$4e^{-8}$ PCR-
#2	**$1e^{1}$ PCR+**	**1 PCR+**	$1e^{-1}$ PCR-	$1e^{-2}$ PCR-	$1e^{-3}$ PCR-	$1e^{-4}$ PCR-	$1e^{-5}$ PCR-	$1e^{-6}$ PCR-	$1e^{-7}$ PCR-	$1e^{-8}$ PCR-
#3	**$2e^{1}$ PCR+**	**2 PCR+**	**$2e^{-1}$ PCR+**	**$2e^{-2}$ PCR+**	**$2e^{-3}$ PCR+**	$2e^{-4}$ PCR-	$2e^{-5}$ PCR-	$2e^{-6}$ PCR-	$2e^{-7}$ PCR-	$2e^{-8}$ PCR-

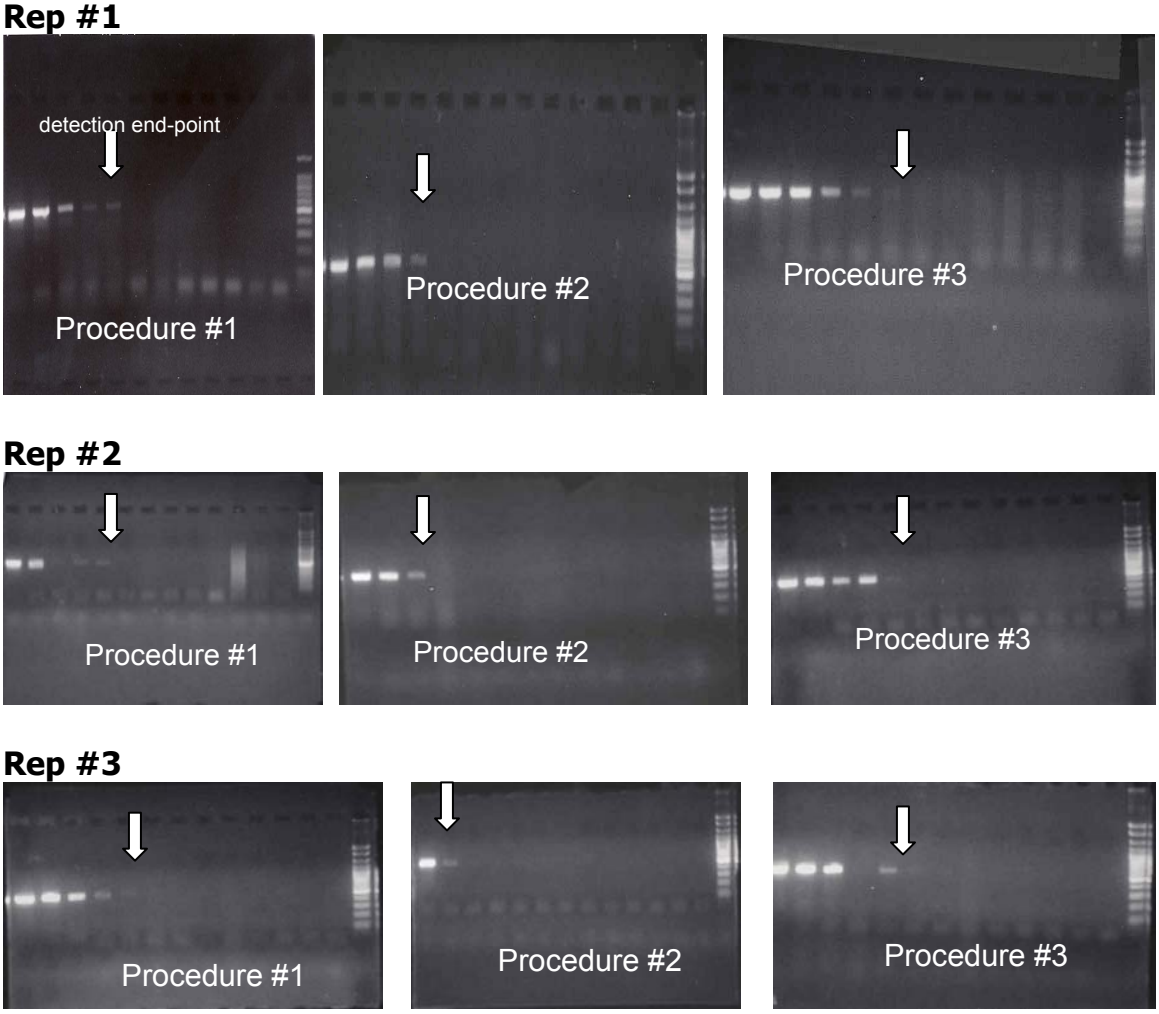

Figure 2. Comparison of PCR detection limits of EtBr-stained agarose gels using 3 different RNA recovery procedures for recovering/detecting PRRSv RNA from inoculated boar semen. Each recovery procedure was evaluated with 10-fold dilutions of cell culture PRRSv that had been inoculated into uninfected boar semen. The RNA was then subjected to PRRSv-specific RT-PCR[1] and interpreted by electrophoresis and visual resolution of the predicted PCR product. The arrow indicates the final dilution where the visible predicted amplicon could be detected. (See Table 1 for interpretations.)

b. SYBR green detection and characterization experiments. An important aspect of this project was the evaluation of SYBR green in *rt*-PCRs to allow not only the "real-time" detection of PRRSv RNA but to also be able to make a rapid assessment of a positive *rt*-PCR by assessing the melting temperatures (T_m) of the PCR products as calculated as the peak of the negative first derivative of the melting curve.

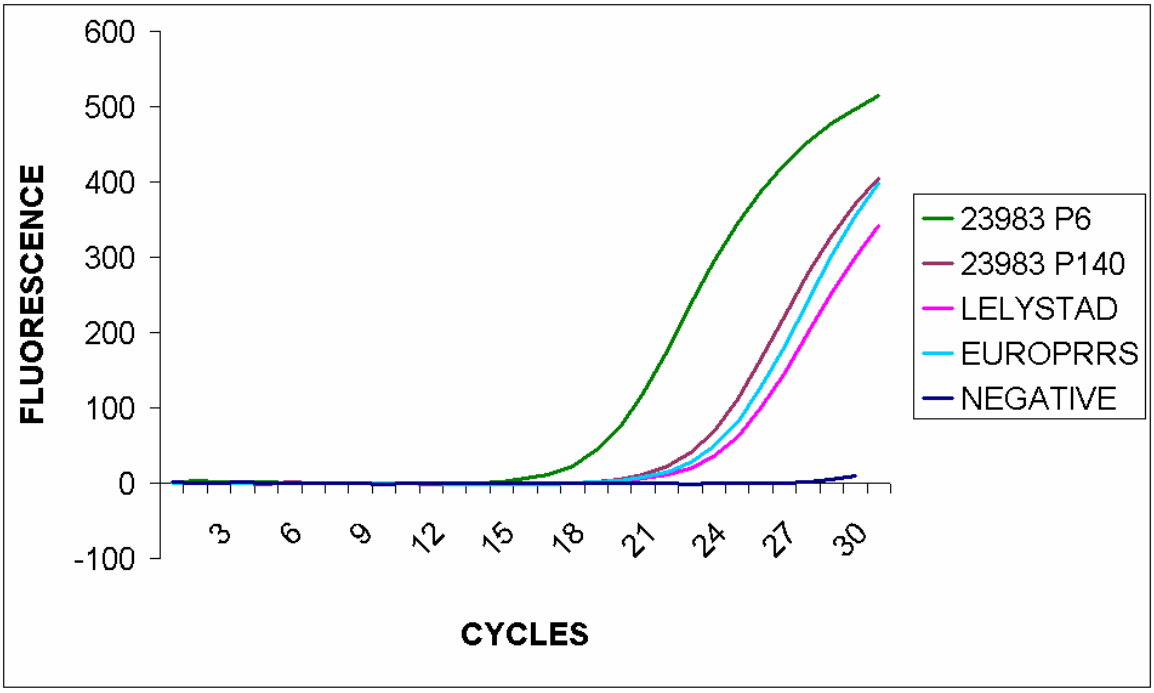

Figure 3. Comparisons of North American and European PRRSv strains in real-time (*rt*)-PCRs using SYBR green in Cepheid's SmartCycler.

Figure 3 demonstrates the effective Sybr green "real-time" (*rt*)-PCR amplification and detection of different PRRSv strains which included a North American PRRSv strain (23983 P6 and 23983 P140) and European PRRSv strains (Lelystad and EuroPRRS). The PCR primers target ORF 7 that codes for the N or nucleocapsid protein and gives a 508bp PCR product with the North American strain 23983 RNA and a 487bp product with the European Lelystad RNA. The melting curve analysis was completed using the first derivative in a SYBR green *rt*-PCR, where the melting temperature (T_m) of the PCR products is calculated as the peak of the negative first derivative of the melt curve. The temperature that that occurs is denoted in red on the graph, and in parenthesis in the legend. All of the RNA used was tissue culture-derived PRRSv RNA. Strain 23983 P140 was used as inoculum in boar semen and recovered using RNA recovery procedure #3 which used bromelain. Two distinct peaks were evident and indicated the ability to use T_m as a method to distinguish North American and European PRRSv strains following *rt*-PCR.

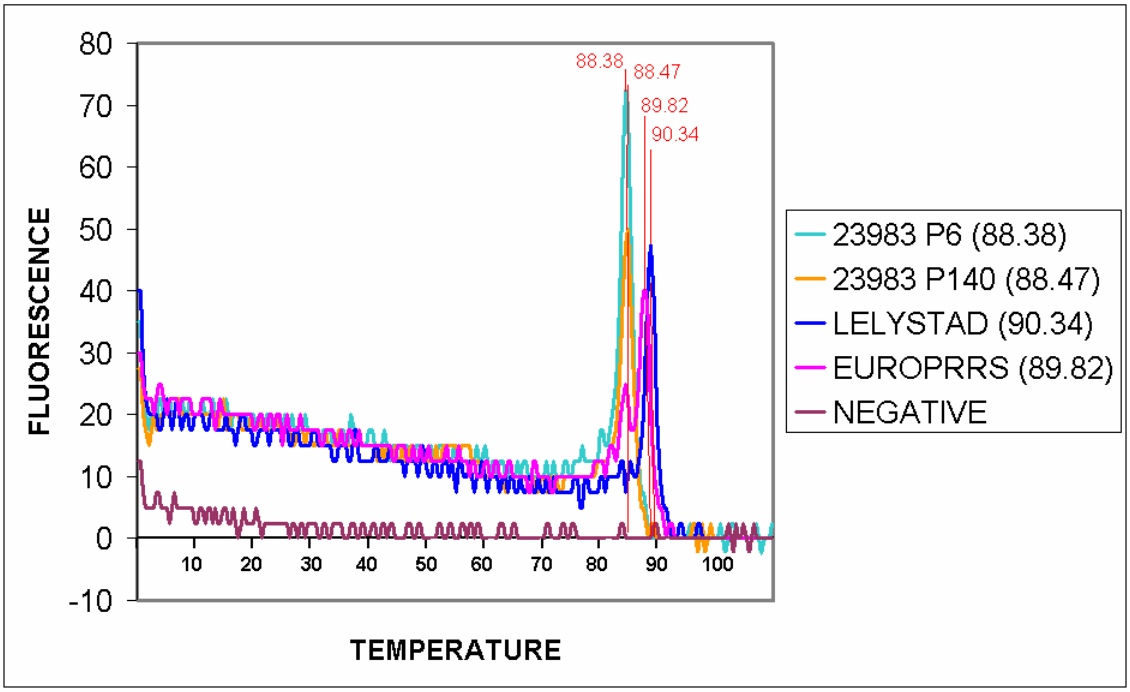

Figure 4. Melting curve characterization of different PRRSv strains using the first derivative of the melt curve in a SYBR green *rt*-PCR. Note that two distinct peaks were evident, indicating the ability to use T_m as a method to distinguish North American and European PRRSv strains.

c. **Infection studies** A total of 6 boars were reared and trained to mount a dummy for collecting semen samples. All boars were serologically evaluated twice prior to challenge using a commercial ELISA kit (IDEXX's HerdCheck ELISA). All boars were serologically PRRSv negative at that time (Tables 2 & 3). Four boars (42-2, 45-1, 47-9, 48-4) were infected intranasally with PRRSv (lab isolate 23983 P6), while two boars (47-4, 50-2) were mock infected and served as negative controls. Blood samples and semen samples were collected once and twice a week respectively, for virus isolation (VI), serology, RNA recover (using procedures #1 and #3 on semen and the Qiagen RNAeasy kit on serum), and three different PCR detection procedures on all RNA samples [SYBR green *rt*-PCR; following a SYBR green *rt*-PCR, the DNA was electrophoresed on agarose gels and stained with ethidium bromide (EtBr) and visualized under UV light (Note: This procedure was referred to as the traditional PCR amplification and detection procedure); and Southern-blot hybridizations using a complimentary oligonucleotide probe to DNA that had been transferred from SYBR green *rt*-PCR gels].

Serology- The mock infected boars never PRRSv-seroconverted using the commercial ELISA during the study (Table 3), but all four PRRSv-challenged boars seroconverted by day after infection (dai)-11 and remained positive through the end of the study at dai-27 (Table 2).

Virus Isolation (VI)- VI was performed on all challenged boar semen and serum samples (Table 4). Boar #42-2 was positive by VI only once from a serum sample collected on dai-5, but was negative on all subsequent serum samples. Boar #42-2 was never PRRSv-positive from a semen sample (Table 4). All remaining PRRSv-challenged boars were positive by VI from serum samples by dai-5. Boar #47-9 demonstrated the longest PRRSv-viremia, being positive by dai-11. Boar #48-4 demonstrated the highest titered serum viremia, demonstrating virus at the 5^4 serum dilution. Three of the 4 boars never demonstrated virus shedding in semen samples as

demonstrated by negative VI attempts (Table 4). Only boar #48-4 demonstrated PRRSv in semen and this was identified only on the dai-13 semen sample.

PCR Results- Three of the 4 boars demonstrated at least one PCR positive result in serum or semen (Table 5). Only boar #42-2 was consistently PCR negative, regardless of the sample type, the RNA recovery procedure used, or the method of detecting the PCR amplicon. This contrasted with the limited detectability of PRRSv RNA by PCR in boar #45-1, which was only PCR positive in the serum sample collected on dai-5 (Table 5). Serum collected on dai-5 from this boar was only PCR positive when detected by EtBr-stained gels or when Southern-blotted and probed with a labeled oligonucleotide. SYBR green *rt*-PCR detection on the dai-5 serum from boar #45-1 was negative. All PCRs from boar #45-1 were negative, regardless of RNA recovery process or detection method used (Table 5).

PCR results from boars #47-9 and #48-4 resulted in the highest number of PRRSv positive interpretations (Table 4). Similar to boar #45-1, both boars #47-9 and 48-4 were PCR-positive in serum samples collected on dai-5 when detected by EtBr-stained gels or when Southern-blotted with a labeled oligonucleotide probe. Viremias were only detectable by PCR in serum samples from boar #47-9 on dai-5, while boar #48-4 was also PCR-positive on dai-11. Serum PCR results were only detected in EtBr-stained gels or when Southern-blotted. In this study no PRRSv-challenged boar demonstrated a PRRSv-positive PCR serum sample when using only SYBR green *rt*-PCR as the detection method. This contrasted with the 100% agreement in test interpretations between serum samples where PRRSv-positive PCR serum samples were detected using electrophoresis and EtBr-staining, or Southern blot DNA hybridizations with a ORF7-specific oligonucleotide probe.

Contrasting with the lack of PCR detectability of PRRSv RNA in semen samples from boars #42-2 and 45-1, boars #47-9 and 48-4 both demonstrated positive semen PCR results commencing on dai-6 and extending to at least dai-13 (Table 5). Both boars were PCR negative by dai-18 and remained so through dai-27. In comparing RNA recovery procedures on these two boars it became apparent that the RNA recovery process #3 was not as effective in recovering RNA for SYBR green *rt*-PCR amplification and detection as was the RNA recovery procedure #1 for PRRSv RT-PCR amplification and detected using electrophoresis and EtBr-staining, or Southern blot DNA hybridizations with a ORF7-specific oligonucleotide probe. Only one semen sample where RNA was recovered using RNA recovery procedure #3 resulted in a positive PCR result and that was from boar #47-9 on dai-6 (Table 5). This was in agreement with the PCR results from the same sample where RNA was recovered with procedure #1 and amplified using PRRSv RT-PCR amplification and detected using electrophoresis and EtBr-staining, or Southern blot DNA hybridizations.

Comparisons between the different detection procedures demonstrated that using a DNA hybridization step following the RT-PCR resulted in a greater detection sensitivity than using either SYBR green in “real-time” PCR assays or electrophoresis and EtBr-staining of RT-PCR assays. This was very evident when PRRSv-positive semen samples on dai-11 could only be detected when Southern-blotted with a labeled DNA probe (Figure 6). SYBR green detection on PRRSv-positive semen samples could not be detected by SYBR green (Figure 5) or by EtBr-stained agarose gels (Figure 6).

Table 2. Serology results of boars that were challenged with PRRSv.
*Serological results were generated on serum samples using the IDEXX HerdCheck ELISA. An ELISA OD reading of >.39 was considered as a positive PRRS ELISA (+), while a reading <.4 was considered as a negative PRRS ELISA (-).

Date	4/13/2004	4/21/2004	4/27/2004	5/3/2004	5/14/2004	5/19/2004
Days Pre (-) or Post (+) Infection	-10	-1	+5	+11	+22	+27
Boar #42-2	.003(-)*	-.111(-)	.013(-)	1.25(+)	2.054(+)	1.953 (+)
Boar #45-1	-.044(-)	-.067(-)	.023 (-)	1.343(+)	1.608(+)	1.586 (+)
Boar #47-9	.09(-)	.09(-)	0.054(-)	.492(+)	1.856(+)	1.231 (+)
Boar #48-4	.057(-)	.013(-)	0.06(-)	1.447(+)	2.318(+)	1.414 (+)

Table 3. Serology results of control boars that were not challenged with PRRSv.

*Serological results were generated on serum samples using the IDEXX HerdCheck ELISA. An ELISA reading of >.39 was considered as a positive PRRS ELISA(+), while a reading <.4 was considered as a negative PRRS ELISA (-).

Date	4/12/2004	4/19/2004	4/26/2004
Boar #47-4	-.067(-)*	-.047(-)	-.023(-)
Boar #50-2	.027(-)	.01(-)	-.023 (-)

Table 4. Virus isolation results from boars experimentally challenged with PRRSv.

Days Post Infection	4	5	6	11	12	13	14	18	20	22	25	27
Boar # 42-2												
VI-Serum		5^3 *		neg						neg		neg
VI-Semen	neg		neg		neg		neg	neg	neg			
Boar # 45-1												
VI-Serum		5^2		neg						neg		neg
VI-Semen	neg		neg	neg		neg		neg	neg		neg	
Boar # 47-9												
VI-Serum		5^2		5^2						neg		neg
VI-Semen	neg		neg	neg		neg		neg	neg		neg	
Boar # 48-4												
VI-Serum		5^4		neg						neg		neg
VI-Semen	neg		neg	neg		5^1		neg	neg		neg	

*Virus isolation was completed on Marc 145 cells making five-fold dilutions of the sample and positive results are reported in final end-point dilution; negative results were reported as neg.

Table 5. Comparison of dai-PCR results from PRRSv-challenged boar serum and semen using different RNA recovery procedures and detection procedures. Evaluation of serum samples for PRRSv RNA using ethidium bromide (EtBr)-stained agarose gels (PCR-Serums), SYBR green *rt*-PCR from serum samples (SYBR-Serum), and Southern-blotted SYBR green *rt*-PCR conclusions (Southern Blot-Serum). Evaluation of boar's semen samples for PRRSv RNA in EtBr-stained agarose gels using RNA recovery process #1 and #3 (PCR-Semen), Sybr green *rt*-PCR from semen samples using RNA recovery process #1 and #3 (SYBR-Semen), and Southern-blotted Sybr green *rt*-PCR conclusions (Southern Blot-Semen) using RNA recovery process #1 and #3.

Boar # 42-2	4/26/2004	4/27/2004	4/28/2004	5/3/2004	5/4/2004	5/6/2004	5/10/2004	5/12/2004	5/14/2004	5/19/2004
Days After Infected	4	5	6	11	12	14	18	20	22	27
PCR-Serum		neg		neg					neg	neg
SYBR-Serum		neg		neg					neg	neg
Southern Blot-Serum		neg		neg					neg	neg
PCR-Semen -RNA #1	neg		neg		neg	neg	neg	neg		
PCR-Semen -RNA #3	neg		neg		neg	neg	neg	neg		
SYBR-Semen -RNA #1	neg		neg		neg	neg	neg	neg		
SYBR-Semen -RNA #3	neg		neg		neg	neg	neg	neg		
Southern Blot-Semen -RNA #1	neg		neg		neg	neg	neg	neg		
Southern Blot-Semen -RNA #3	neg		neg		neg	neg	neg	neg		

Boar # 45-1	4/26/2004	4/27/2004	4/28/2004	5/3/2004	5/5/2004	5/10/2004	5/12/2004	5/14/2004	5/17/2004	5/19/2004
Days After Infected	**4**	**5**	**6**	**11**	**13**	**18**	**20**	**22**	**25**	**27**
PCR-Serum		**pos**		neg				neg		neg
SYBR-Serum		neg		neg				neg		neg
Southern Blot-Serum		**pos**		neg				neg		neg
PCR-Semen -RNA #1	neg		neg	neg	neg	neg	neg		neg	
PCR-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	
SYBR-Semen -RNA #1	neg		neg	neg	neg	neg	neg		neg	
SYBR-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	
Southern Blot-Semen -RNA #1	neg		neg	pos	neg	neg	neg		neg	
Southern Blot-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	

Table 5. (Continued)

Boar # 47-9	4/26/2004	4/27/2004	4/28/2004	5/3/2004	5/5/2004	5/10/2004	5/12/2004	5/14/2004	5/17/2004	5/19/2004
Days After Infected	**4**	**5**	**6**	**11**	**13**	**18**	**20**	**22**	**25**	**27**
PCR-Serum		**pos**		neg				neg		neg
SYBR-Serum		neg		neg				neg		neg
Southern Blot-Serum		**pos**		neg				neg		neg
PCR-Semen -RNA #1	neg		**pos**	**pos**	**pos**	neg	neg		neg	
PCR-Semen -RNA #3	neg		**pos**	neg	neg	neg	neg		neg	
SYBR-Semen -RNA #1	neg		neg	neg	neg	neg	neg		neg	
SYBR-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	
Southern Blot-Semen -RNA #1	neg		**pos**	**pos**	**pos**	neg	neg		neg	
Southern Blot-Semen -RNA #3	neg		**pos**	neg	neg	neg	neg		neg	

Boar # 48-4	4/26/2004	4/27/2004	4/28/2004	5/3/2004	5/5/2004	5/10/2004	5/12/2004	5/14/2004	5/17/2004	5/19/2004
Days After Infected	**4**	**5**	**6**	**11**	**13**	**18**	**20**	**22**	**25**	**27**
PCR-Serum		**pos**		**pos**				neg		neg
SYBR-Serum		neg		neg				neg		neg
Southern Blot-Serum		**pos**		**pos**				neg		neg
PCR-Semen -RNA #1	neg		**pos**	**pos**	**pos**	neg	neg		neg	
PCR-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	
SYBR-Semen -RNA #1	neg		neg	neg	neg	neg	neg		neg	
SYBR-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	
Southern Blot-Semen -RNA #1	neg		**pos**	**pos**	**pos**	neg	neg		neg	
Southern Blot-Semen -RNA #3	neg		neg	neg	neg	neg	neg		neg	

Figure 5. Melting curve characterization of semen SYBR green *rt*-PCR results from different PRRSv strains using the first derivative of the melt curve in a SYBR green *rt*-PCR. Only one sample indicated a positive SYBR green *rt*-PCR melting curve and this was indicated in the positive PRRSv RNA control (+) and resulted in a T_m of 84.31°C. Legend: A1, 42-2 MN, SYBR green *rt*-PCR result of semen sample from boar 42-2 collected day after infection (dai)-12 with RNA recovered using procedure #1; A2, 42-2 BR, SYBR green *rt*-PCR result of semen sample from boar 42-2 collected dai-12 with RNA recovered using procedure #3; A3, 45-1 MN, SYBR green *rt*-PCR result of semen sample from boar 45-1 collected dai-11 with RNA recovered using procedure #1; A4, 45-1 BR, SYBR green *rt*-PCR result of semen sample from boar 45-1 collected dai-11 with RNA recovered using procedure #3; A5, 47-9 MN, SYBR green *rt*-PCR result of semen sample from boar 47-9 collected dai-11 with RNA recovered using procedure #1; A6, 47-9 BR, SYBR green *rt*-PCR result of semen sample from boar 47-9 collected dai-11 with RNA recovered using procedure #3; A7, 48-4 MN, SYBR green *rt*-PCR result of semen sample from boar 48-4 collected dai-11 with RNA recovered using procedure #1;; A8, 48-4 BR, SYBR green *rt*-PCR result of semen sample from boar 48-4 collected dai-11 with RNA recovered using procedure #3; A9, TE buffer negative PRRSv control; A10, positive (+) PRRSv RNA control.

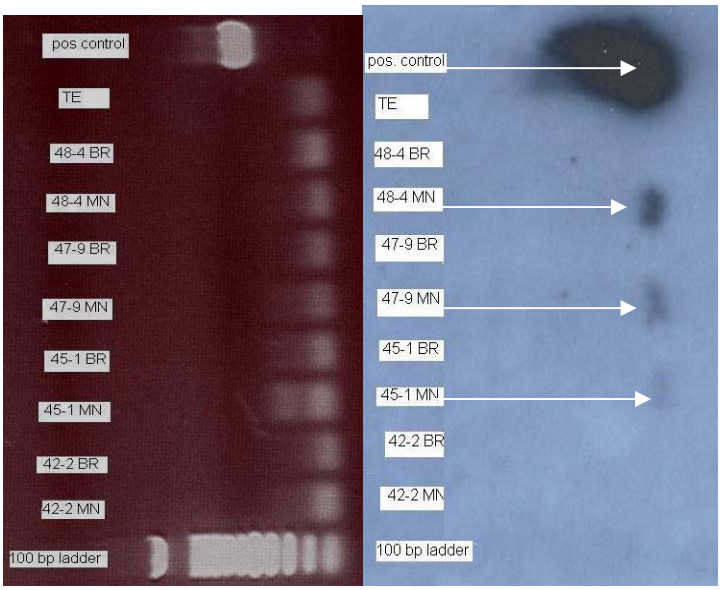

Figure 6. Comparison of PCR results of boar semen samples using RNA recovery procedures #1 and #3 by agarose gel electrophoresis and ethidium bromide staining, and by Southern blot hybridizations. The left portion of figure is an ethidium bromide-stained agarose gel and the right portion is the Southern blot of that gel following DNA hybridization with an oligonucleotide probe. Legend: Arrow indicates positive hybridization results; Pos control, positive PRRSv RNA control; TE, TE buffer negative PRRSv control; 48-4 MN, semen sample from boar 48-4 day 11 after infection with RNA recovered using procedure #1; 48-4 BR, semen sample from boar 48-4 day 11 after infection with RNA recovered using procedure #3; other sample follow similar labeling pattern on boars 47-9, 45-1. Boar 42-2 samples were collected 12 days post-infection; 100 bp ladder, electrophoretic marker.

Discussion: Our initial evaluations using PRRSv-inoculated semen indicated that detection of PRRSv RNA in semen was possible using SYBR green and that the resulting PCR product and its T_m could be used to characterize the strain of PRRSv. Unfortunately, in challenge studies the sensitivity of the SYBR green detection was insufficient to detect PRRSv RNA in infected boar semen or serum. Incorporating a DNA hybridization step to the *rt*-PCR appears to be a logical choice for improving sensitivity. This might include the incorporation of either TaqMan or Molecular Beacon fluorescent-labeled DNA probes.

Neither RNA recovery procedures #1 or #3 required as many manipulations as procedure #2 when using PRRSv-inoculated semen. We were initially encouraged that procedure #3 might allow the successful detection of lower concentrations of PRRSv. Because procedure #3 involved the 1:1 dilution of the semen sample with the bromelain solution, improved sensitivity potentially could be obtained by reducing the volume of bromelain solution that is added to each semen sample. Regardless, the improved recovery of PRRSv RNA with pretreatment of bromelain could not be demonstrated on naturally infected semen. Several reasons may contribute to this. One, the dilution of bromelain solution may dilute out any beneficial effects of the enzymatic action, or that PRRSv may be more cellular-associated and require further enzymatic action other than that provided by the bromelain solution. Clearly, additional research will be necessary to optimize the PRRSv RNA recovery process from PRRSv-infected semen as this is an important component of any automated nucleic acid-based assay. We recommend that the goal continue to be the identification of the best RNA recovery process for semen, with an additional goal being that the process be straight-forward, user-friendly and adaptable so that the process could be totally automated and self-contained for on-farm evaluations.

The challenge studies highlight the need for improving our PRRSv diagnostic approaches. Correlating the current PRRSv infectious status of a boar using only one diagnostic approach from only one type of sample will result in misdiagnoses. As was demonstrated in this study, all boars seroconverted to PRRSv using a commercial ELISA indicating past-exposure to PRRSv antigens but these tests indicated nothing about the current status of a PRRSv infection as none of the boars seroconverted until sometime between dai-5 and dai-11. Similarly, only one boar on one sampling date (dai-13) demonstrated PRRSv in semen by traditional tissue culture procedures for virus isolation. Virus was detectable in all boars in serum samples by virus isolation, however these were only detectable by dai-5 and the viremias were short-lived as only one boar was viremic by dai-13.

Comparing different RNA recovery procedures on different boar samples also demonstrated the potential for misdiagnoses. No sample where RNA recovery procedure #3 was used resulted in a positive PCR interpretation, regardless of the method of detecting/interpreting the PCR. This contrasted with RNA recovery procedure #1 where EtBr-stained gel and Southern blot detection approaches resulted in positive PCR interpretations. Additional RNA recovery procedures will need to be evaluated in the future as it is clear following the experimentation using PRRSv-inoculated or spiked semen samples that this approach is not an effective method of comparing PRRSv RNA recovery from natural infections. Optimizing PRRSv RNA recovery will therefore require additional challenge studies.

The original intent of this study was to evaluate the efficiency of a SYBR green "real-time" PCR for detecting PRRSv in boar semen and serum. Unfortunately, when the SYBR green PCR was compared to PCR results detected with electrophoretic gels stained with EtBr or Southern blotted and hybridized with a DNA probe, the SYBR green PCR was unable to detect the presence of PRRSv RNA in all serum and semen samples evaluated. While the gels stained with EtBr were superior to the SYBR green PCR results, the Southern blots on the same PCRs were able to detect PRRSv RNA on samples that were otherwise undetectable. The need for a DNA hybridization step in any PCR assay for detecting PRRSv RNA in serum or semen cannot be overstated based on these results and the potential for false-negative misdiagnoses. To overcome this, we recommend the development and evaluation of PCR approaches that incorporate automated DNA hybridization steps into the PCR analysis, such as TaqMan or Molecular Beacon detection systems. These fluorgenic-based PCR assays would allow for improvement in "real-time" detection sensitivity and specificity that is lacking with the SYBR green detection system.

Lay Interpretation: The detection of PRRSv in boars is critical for developing and maintaining a biosecure breeding program, however it is complicated by many variables. Correlating the current PRRSv infectious status of a boar using only one diagnostic approach at one point in time from only one type of sample can result in misdiagnoses. As was demonstrated in this study, all boars seroconverted to PRRSv using a commercial ELISA indicating past-exposure to PRRSv antigens, but only one boar on one sampling date (day after infection, [dai-13]) demonstrated PRRSv in semen by traditional tissue culture procedures for recovering the virus. Virus was detectable in all boars in serum samples by virus isolation, however these were only detectable by dai-5 and the viremias were short-lived as only one boar was viremic by dai-13. Comparing different PCR detection procedures on these different boar samples also demonstrated the potential for misdiagnoses.

The original intent of this study was to evaluate the efficiency of a SYBR green "real-time" PCR for detecting PRRSv in boar semen and serum. Unfortunately, when the

SYBR green PCR was compared to PCR results detected with electrophoretic gels stained with ethidium bromide (EtBr) or blotted and hybridized with a DNA probe (Southern blots), the SYBR green PCR was unable to detect the presence of PRRSv RNA in all serum and semen samples evaluated. While the gels stained with EtBr were superior to the SYBR green PCR results for detecting PRRSv in serum and semen, the Southern blots on the same PCRs were able to detect PRRSv RNA on samples that were otherwise undetectable.

The need for a DNA hybridization step in any PCR assay for detecting PRRSv RNA in serum or semen cannot be overstated based on these results and the potential for misdiagnoses, particularly false-negative PCR test results. To overcome this, we recommend the development and evaluation of PCR approaches that incorporate automated DNA hybridization steps into the PCR analysis, such as TaqMan or Molecular Beacon detection systems. These fluorgenic-based PCR assays will allow for improvements in detection sensitivity and specificity that is lacking with the SYBR green detection system, but also allow for rapid "real-time" (*rt*)-PCR to be applied. Also recognized in this study were the importance of effective RNA recovery procedures from semen samples and their importance to any sensitive PRRSv PCR detection procedure. Additional research will be required to identify and optimize the most efficient PRRSv RNA recovery processes from semen and other tissues. The goal of the process should be the development of a straight-forward, user-friendly process that can be totally automated, self-contained PRRSv-RNA detection system.

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