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Comparison of Commercial Real-Time Reverse Transcription-PCR Assays for Reliable, Early, and Rapid Detection of Heterologous Strains of Porcine Reproductive and Respiratory Syndrome Virus in Experimentally Infected or Noninfected Boars by Use of Different Sample Types

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The aims of this study were to compare three commercial porcine reproductive and respiratory syndrome virus (PRRSV) real-time reverse transcription-PCR (RT-PCR) assays for detection of genetically diverse PRRSV isolates in serum, semen, blood swabs, and oral fluids collected from experimentally infected boars and to evaluate the effects of sample pooling. Six groups of three boars negative for PRRSV were each inoculated with one of six PRRSV isolates (sharing 55 to 99% nucleotide sequence identity in ORF5). Samples were collected on days −2, 1, 3, 5, 7, 14, and 21 postinoculation (p.i.) and tested by one of three commercially available real-time RT-PCR assays (VetMax from Applied Biosystems, Foster City, CA [abbreviated AB]; VetAlert from Tetracore, Rockville, MD [TC]; and AcuPig from AnDiaTec GmbH, Kornwestheim, Germany [AD]). At day 1 p.i., all assays detected at least one positive sample in each group. The highest detection rates were on days 3 and 5 p.i. Between days 1 and 7 p.i., serum samples had the highest detection rate (90%) with 100% agreement between tests, followed by blood swabs (kappa value of 0.97) and semen (kappa value of 0.80). Oral fluids had the lowest detection rates (AB, 55%; TC, 41%; AD, 46%) and the highest disagreement between kits (kappa value of 0.63). Pools of five samples did not reduce the detection rates if there was one positive sample with a large amount (cycle threshold, <30) of viral RNA in the pool. Serum and blood swab samples had shorter turnaround times for RNA extraction. The AB assay had a 1.6-times-shorter PCR time. In summary, serum and blood swabs had the best performance with highest detection rates and agreement between assays and the shortest turnaround times.

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to be the most important pathogen affecting pigs in North America (1). PRRSV is a small, enveloped, single-stranded positive-sense RNA virus of the family *Arteriviridae* and can be divided into two genotypes: type 1 (European type [EU]) and type 2 (North American type [NA]) (2). Although a variety of commercial vaccines are available on the global market, the virus remains difficult to control, and the demand for PRRSV-naïve replacement genetics and, with it, the need for highly sensitive and specific assays that can detect genetically diverse strains and provide information on the most appropriate samples for testing continue to grow.

Presently, many boar studs in the United States are PRRSV negative and are routinely tested for PRRSV to ensure that PRRSV-free semen is used in breeding herds for artificial insemination (1). If previously negative boar studs become infected with PRRSV, it is critical to detect the virus as soon as possible so that any shipments of possible PRRSV-contaminated semen can be stopped.

The reverse transcription-PCR (RT-PCR) method in the real-time format is one of the most commonly used techniques for detection of PRRSV RNA because of its sensitivity and specificity and relatively short test turnaround time. However, the high mutation rate, rapid evolution, and genetic variability of PRRSV strains complicate the development of long-term reliable diagnostic assays, and consequently cases of false-negative results with commercially available assays have been reported (3–5).

Active PRRSV surveillance in boar studs relies mainly on collection and testing of serum, semen, blood swabs, and, more recently, oral fluids (6, 7). The choice of sample type should take into consideration the availability and ease of collection in addition to the sensitivity and specificity of the RT-PCR assay. Studies have shown that PRRSV RNA can be detected in boar serum, oral fluids, and blood swabs as early as 24 to 48 h postinfection and in semen samples as early as 48 to 120 h postinfection (6–9). Isolate-specific differences in the levels of PRRSV replication and shedding in the host have been reported (10), and as veterinarians and diagnosticians consider using alternative sampling methods, it is important to conduct an unbiased comparison of the ability of different commercial real-time RT-PCR tests to detect genetically diverse isolates of PRRSV in new and conventional sample types.

The sample collection process, transport, and testing are time-consuming and labor-intensive. In order to test a large number of animals and reduce cost, pooled sample analysis has been used

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TABLE 1 Experimental design

Group no.	No. of boars	PRRSV isolate	PRRSV type	Yr of isolation	Reference or source
1	3	VR2385	2	1991	13
2	3	SDSU73	2	1996	14
3	3	JA142	2	1996	14
4	3	FL12	2	2004	17
5	3	2010011381	1	2010	This manuscript
6	3	NC16865	2	2006	15

successfully in recent years for detection and surveillance of infectious diseases (9, 11, 12). Pooling of serum or blood swabs is used on a regular basis by many boar stud owners to monitor PRRSV status by RT-PCR. While a single study demonstrated a decrease in sensitivity, especially during the first days of PRRSV infection, when pools of three and five samples were used in serum and blood samples (9), the effects of processing and analyzing samples individually or pooled have never been comprehensively compared.

A complete understanding of the sensitivity and specificity of the test used to detect PRRSV RNA in a variety of samples will better inform decisions on boar stud PRRSV monitoring protocols. The aims of this study were (i) to compare the sensitivities and specificities of three commercially available PRRSV diagnostic assays to detect genetically diverse isolates of PRRSV in different sample types (serum, semen, blood swabs, and oral fluids), (ii) to evaluate the effects of pooling serum and blood swab samples on diagnostic accuracy, and (iii) to compare the turnaround times of the three assays.

MATERIALS AND METHODS

Experimental samples. (i) Animals and housing. Eighteen 6-month-old boars were acquired from a commercial PRRSV naïve breeding herd. Upon arrival at the Iowa State University Livestock Infectious Disease Isolation Facility, the boars were randomly divided into six groups of three boars and housed in separate rooms, each containing three individual pens for housing of the boars and a collection pen within the same room.

(ii) Experimental design, sample collection, and storage. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. After a short acclimation period, the boars were infected with one of six different PRRSV isolates as summarized in Table 1. Two days preinoculation and at 1, 3, 5, 7, 14, and 21 days postinoculation (dpi), serum, semen, blood swabs, and oral fluids were collected. For sample collection, individual animals were moved into a collection pen, and semen was collected using the gloved-hand technique. Concurrently, blood was collected by venipuncture of the saphena vein in 8.5-ml serum separator tubes (BD Vacutainer; BD Biosciences). Immediately after blood collection, blood drops were collected from the surface of the skin using a polyester swab as described previously (7). Oral fluids were collected individually from each boar by using a cotton rope as described previously (16). All samples were stored on ice after collection until arrival at the laboratory. Serum and oral fluid samples were centrifuged at 1,000 × *g* for 10 min and aliquoted into 5-ml plastic tubes. Semen was aliquoted in 1.5-ml plastic microtubes. Samples were stored at −80°C until tested.

(iii) Inocula and inoculation. Type 2 PRRSV isolate VR2385 was recovered from a herd located in southwestern Iowa that had experienced severe respiratory disease in 3- to 16-week-old pigs and high numbers of late-term abortions in 1991 (13); highly virulent type 2 PRRSV isolate SDSU73 was recovered from a sow herd that experienced a severe epidemic of atypical PRRS in 1996 (14); type 2 PRRSV isolate JA142 was recovered from a herd that experienced a severe epidemic of acute PRRS in 1996 (14); the type 2 FL12 PRRSV isolate (17) was obtained from PRRSV NVSL 97-7895 isolated in 1997 in southeastern Iowa from a herd experiencing severe reproductive failure in pregnant sows (18); type 1 PRRSV isolate ISU-2010011381 was recovered from a swine farm in Iowa in 2010 (unpublished data); and type 2 PRRSV NC16845b was recovered from finisher pigs experiencing high mortality and morbidity in North Carolina in 2006 (15). All PRRSVs were propagated at low passage numbers in MARC-145 cells, and the infectious titer of each virus stock was determined in MARC-145 cells by an immunofluorescence assay (IFA) as described previously (19). Experimental inoculation was done by slowly dripping 4 ml of the inoculum containing a 50% tissue culture infective dose ($TCID_{50}$) of $10^{4.5}$ into the nostrils.

Field samples. A total of 200 serum and 200 oral fluid samples from individual boars from 22 different herds were selected from routine submissions from known PRRSV-negative herds to the Veterinary Diagnostic Laboratory at Iowa State University. Samples were stored at −80°C until tested.

PRRSV antibody ELISA. Serum samples were tested for anti-PRRSV antibodies using a commercial enzyme-linked immunosorbent assay (ELISA) (HerdChek PRRS X3; Idexx Laboratories, Inc., Westbrook, ME) according to the manufacturer's label instructions. Samples were considered positive if the calculated sample-to-positive (S/P) ratio was equal to 0.4 or greater.

RNA extraction. Total nucleic acids of serum and blood swab samples were extracted by using a KingFisher Flex 96-tip comb from Thermo Scientific using a MagMAX-96 viral RNA isolation kit (Ambion) according to the manufacturer's directions. Prior to automated extraction, semen samples were centrifuged at 1,000 × *g* for 10 min, and the cell pellets were resuspended in 300 μl of physiological saline. Oral fluid and semen samples were extracted as previously described (6). Negative controls, using water as a sample, and positive controls, using cell extracts infected with PRRSV type 1 or type 2, were added in each extraction plate.

Real-time RT-PCR. Real-time RT-PCR was performed on RNA extracts using VetMax NA and EU PRRSV reagents (abbreviated here as AB for Applied Biosystems, Foster City, CA), VetAlert NA and EU PRRSV PCR reagents (abbreviated here as TC for Tetracore, Rockville, MD), and AcuPig PRRSV real-time PCR reagents (abbreviated here as AD for AnDiaTec GmbH, Kornwestheim, Germany). Reactions were performed according to the manufacturer's instructions using a 7500 Fast Real-Time PCR System (ABI, Foster City, CA). All three assays were performed on the same day, and the same nucleic acid extract was utilized. For the AB test, thresholds for NA and EU PRRSVs were set at 0.10 and 0.05, respectively. For the TC test, thresholds for NA and EU PRRSVs were set at 145,000 and 51,000, respectively. For the AD test, thresholds for PRRSV were set at 161,000. Based on the cutoff that is currently used by the Veterinary Diagnostic Laboratory at Iowa State University, a cycle threshold (C_T) of <37 cycles was used for considering samples positive for all three assays. If a sample produced a C_T between the established cutoff of 37 and a C_T of 40, it was retested up to two times. If the second result was higher than the threshold of 37 or produced no amplification, the result was considered negative. If the second result was a C_T below the cutoff, then the sample was retested again, and the two agreeing results (negative or positive) for the three tests were considered the final result. Quality control of the real-time RT-PCR process included negative (nuclease-free water) and positive (PRRSV RNA) controls. A negative control without RNA and a positive control provided in each kit were added to each PCR plate. Internal control amplification was evaluated to validate the result as recommended by the manufacturer's protocols. Discrepant results, defined as a sample that exhibited a positive result in one assay and a negative result in another assay, were retested with all three assays. The final result for a given assay was based on an analysis of the initial test result and one

or two repeat test results obtained by each separate assay. If the initial test result was positive for a given assay and one of the retests was positive, the final result was reported as positive for that assay. Conversely, if the initial test result was negative for a given assay and one of the retests was negative, the final result was reported as negative for a given assay.

Sequencing. The open reading frame 5 (ORF5) gene fragment amplified from a PRRSV-positive pig in each group on day 21 postinoculation (p.i.) (groups 1 to 5) or day 7 p.i. (group 6) and the inocula used for the experimental infections were sequenced. Sequencing for ORF5 was also attempted on field samples with repeated discrepant results by real-time RT-PCR. Single-step RT-PCR was performed using a Qiagen One-Step RT-PCR Kit. Each reaction mixture included a 1.0 μM concentration of the primer pair GP5F (5′-ATGTTGGGGAAATGCTTGACCG-3′) and GP5R (5′-CTAAGGACGACTCCATTGTTCCG-3′) (strains VR2385, SDSU73, JA142, and FL12 and field samples positive for PRRSV type 2) or the pair NGP5F (5′-AAGGTGGTATTCGGCAATGTGTC-3′) and NGP5F (5′-GAGGTGATGAACCTCCAGGTTTCTA-3′) (strain NC16845b and field samples positive for PRRSV type 2) or the pair EU-F (5′-TGAGGTGGGCTACAACCATT-3′) and EU-R (5′-AGGCTAGCAC GAGCTTTTGT-3′) (strain 2010011381 and field samples positive for PRRSV type 1) plus 5 μl of 5× RT-PCR master mix, 1.0 mM deoxynucleoside triphosphate (dNTP) mix, 1.5 mM $MgCl_2$, 1.0 μl of RT enzyme mix, and 5 μl of RNA in a 25-μl total reaction volume. Cycling conditions consisted of 30 min at 50°C and 15 min at 95°C, followed by 45 cycles of 94°C for 15 s, 56°C for 30 s, and 68°C for 1 min, with a final extension at 72°C for 10 min. Reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR products were sequenced at the Iowa State University DNA Facility, Ames, IA. Sequences were aligned with published data using BLAST at the National Centre for Biotechnology Information (NCBI [http://www.ncbi.nlm.nih.gov/]). Sequences were compiled using Lasergene software and the Clustal V alignment algorithm (DNAStar, Madison, WI).

Pooling strategy. Serum and blood swab samples individually tested were classified as a high positive when the C_T was ≤29.9, a moderate positive when the C_T was between 30.0 and 34.9, and a low positive when the C_T was between 35.0 and 37.0. One sample of each positive range per virus isolate was selected and diluted in an appropriate amount of negative-control sample of serum or blood swabs to simulate pools of 2, 3, 5, and 10 samples that contain 1 positive sample and 1, 2, 4, and 9 negative samples, respectively.

Turnaround time. The turnaround time of each assay was calculated manually by recording specific time points for sample preparation, the extraction protocol, and the real-time PCR cycle conditions of each set of reagents.

Statistical analysis. Cochran's Q test for matched data, followed by McNemar's test for pairwise comparisons, was used to determine whether the proportions of RT-PCR-positive samples were significantly different by assays or sample type. Differences between groups were considered significant at a P value of <0.05. A kappa index was performed to determine the agreement of positive/negative results between assays and samples types. The strength of agreement was considered as previously described (27): ≤0, poor; 0.01 to 0.2, slight; 0.21 to 0.4, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; and 0.81 to 1, almost perfect. Statistical analyses were performed using SAS, version 9.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Experimental sample collection. All boars were successfully sampled as scheduled. Although the boars showed an overall low interest in chewing the rope, especially during the first week of the trial, the volume of oral fluid collected was sufficient for the analysis.

Confirmation of PRRSV infection. (i) Experimental samples. To determine whether the experimentally inoculated boars had prior exposure to PRRSV and to assess the experimental PRRSV infection, all serum samples were tested for the presence of anti-PRRSV-negative antibodies, and all sera, blood swabs, semen samples, and oral fluid samples on day −2 p.i. were tested by all three PCR assays prior to experimental infection. All serum samples collected on day −2 p.i. tested negative for anti-PRRSV antibodies, and all samples were PRRSV RNA negative by the three assays. All 15 animals in groups 1 to 5 seroconverted between days 7 and 14 p.i. All three boars in group 6 remained serologically negative for PRRSV during the trial (data not shown). However, TC and AD assays detected positive results in one serum and one blood swab sample of one of three boars in this group on day 7 p.i., with C_Ts ranging from 30.4 to 36.7. Sequencing of these samples revealed nonspecific amplifications. All other samples from this group tested negative by all three assays. Based on the negative ELISA and real-time RT-PCR results, group 6 boars were considered noninfected. Sequencing of the ORF5 gene of PRRSV and comparison with the original inocula confirmed that the correct PRRSV isolates were present in the other five experimental groups (data not shown) and that cross-contamination of groups had not occurred.

(ii) Field samples. All field samples utilized were negative for anti-PRRSV antibodies as determined by ELISA.

PRRSV detection by the three commercial real-time assays in experimental samples. To obtain the prevalence of PRRSV RNA detection in each sample type over time, only data from the 15 infected boars (groups 1 to 5) were used. The positive detection rate for each sample type and day postinoculation was calculated by taking the total number of PCR-positive samples in groups 1 through 5, dividing by 15, and multiplying by 100. At day 1 p.i., all assays were able to detect at least one positive sample in each group, with the highest detection rates on days 3 and 5 p.i. for all three assays. On days 14 and 21 p.i., PRRSV RNA detection rates decreased in all sample types except for oral fluids, which coincided with the detection of anti-PRRSV antibodies. The prevalence rates of PRRSV RNA detection in each sample type for each assay are summarized below and in Fig. 1.

(i) Serum samples. Among the sample types, the ability to detect PRRSV RNA was highest in serum samples throughout the study, reaching a positive detection rate of 90% (54/60) during the acute phase of infection (days 1 through 7 p.i.) with 100% agreement among the three assays tested. Among the five groups, group 1 was the only one without any positive detection in serum samples at day 1 p.i. Overall, for the length of the experiment, there was no difference in detection rates between the different commercial assays ($\kappa = 0.94$, $P = 0.48$).

(ii) Blood swabs. PRRSV RNA detection rates in blood swab samples were similar to those in serum samples throughout the study ($\kappa = 0.73$, $P = 0.11$) (Fig. 1). Among the five groups, group 1 was the only one without any positive PRRSV RNA detection in blood swab samples at day 1 p.i. Considering the acute phase of infection (days 1 through 7 p.i.), PRRSV RNA was detected in 86.6% (AB), 86.6% (TC), or 81.6% (AD) of the samples, with nearly complete agreement between the tested assays ($\kappa = 0.97$). The AB and TC tests had higher detection rates in blood swabs samples than the AD test on day 21 p.i. ($P = 0.04$).

(iii) Oral fluids. Oral fluid samples had the lowest detection rate and the highest disagreement between assays throughout the study ($\kappa = 0.40$ to 0.73), with 55% (AB), 41% (TC), and 46% (AD) rates of positive detection from days 1 through 7 p.i. In contrast to the other sample types that showed a decreasing trend

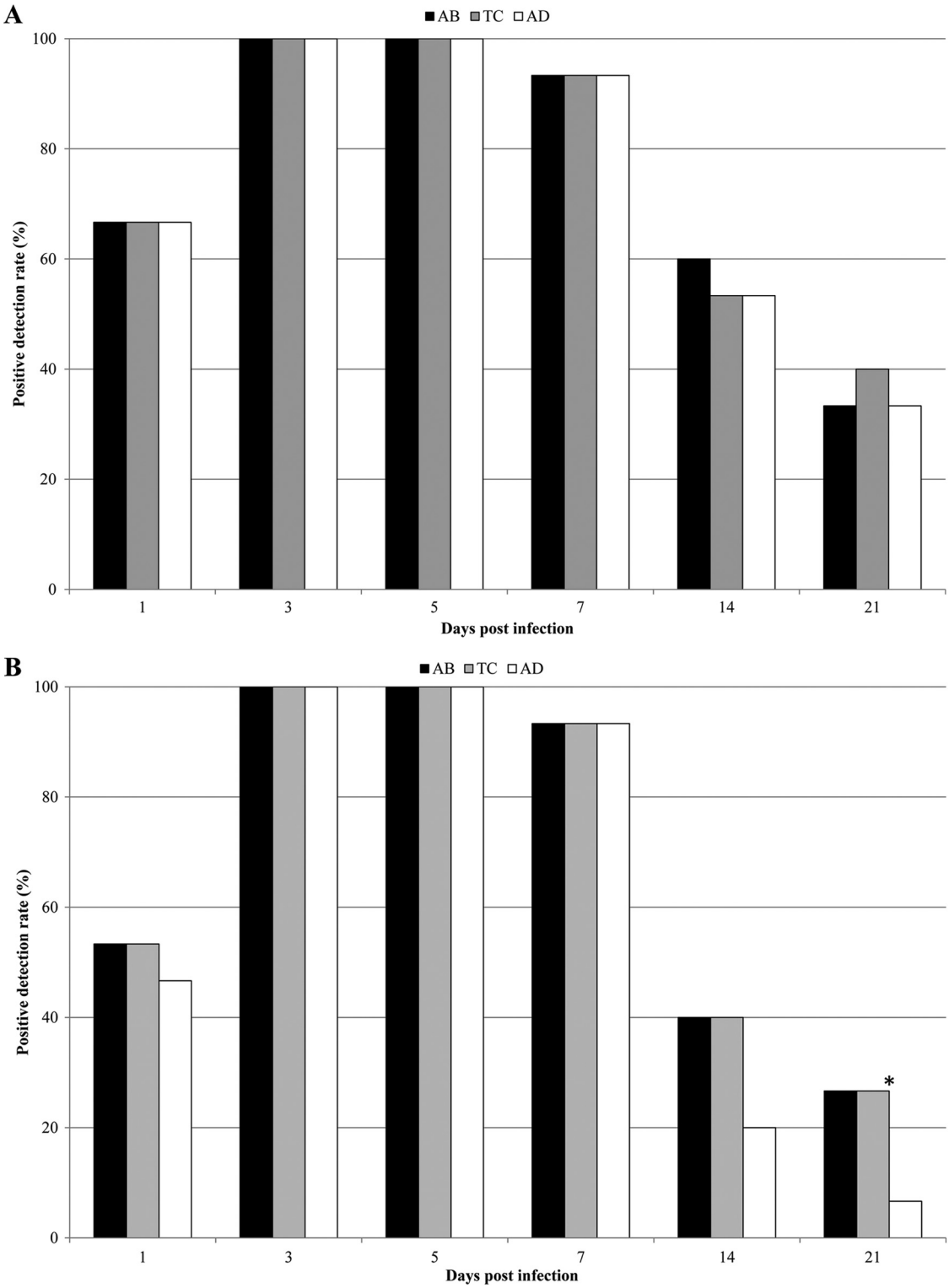

FIG 1 Detection rates for PRRSV RNA in serum (A), blood swab (B), oral fluid (C), and semen (D) samples of 15 experimentally infected boars on days 1, 3, 5, 7, 14, and 21 postinfection by three real-time RT-PCR tests (AB, TC, and AD). Asterisks indicate differences between assays (McNemar's test, $P < 0.05$).

in PRRSV RNA detection after day 7 p.i., there was an increase in detection rates of PRRSV RNA in oral fluids on days 14 and 21 p.i., showing the highest detection rates among sample types on day 21 p.i. Among all tested samples, the TC assay had the lowest detection rate (37/90, or 41.1%; $P = 0.02$). There was no difference between the AB and the AD assays (51/90, or 56.7%, versus 45/90, or 50.0%; $P = 0.06$). Oral fluids had slight agreement with detection rates in serum samples throughout the study ($\kappa = 0.10$, $P = 0.01$).

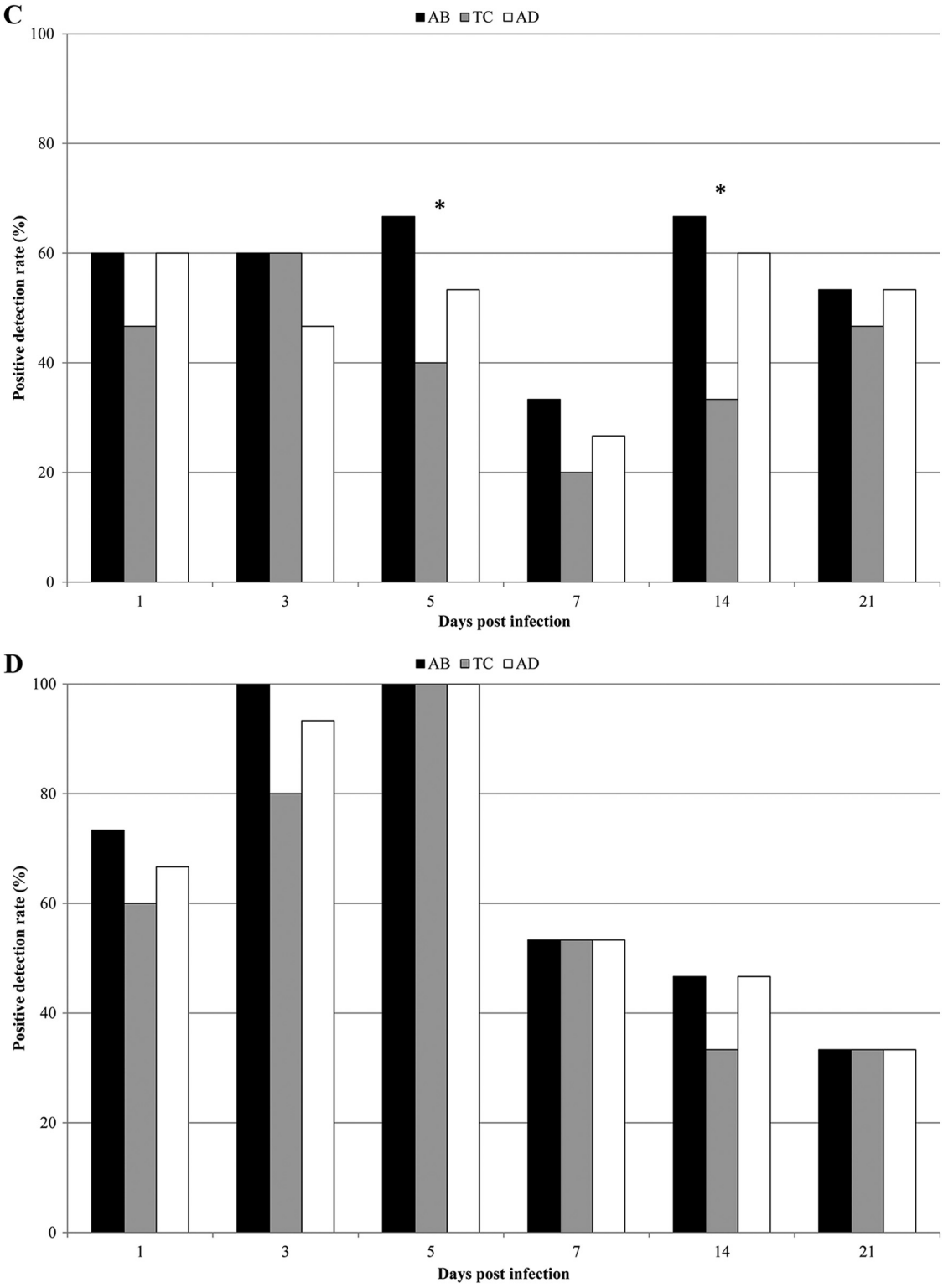

FIG 1 Continued

(iv) Semen samples. During the acute phase of infection (days 1 through 7 p.i.), the detection rates in semen samples were 81.6% (AB), 73.3% (TC), and 78.3% (AD). All groups except group 4 had at least one PRRSV RNA-positive semen sample at day 1 p.i. The AB test had a higher detection rate than TC (61/90, or 67.7%, versus 54/90, or 60.0%; $\kappa = 0.83$, $P = 0.02$). There was no difference between AB and AD assays (61/90, or 67.7%, versus 59/90, or 65.50%; $P = 0.61$).

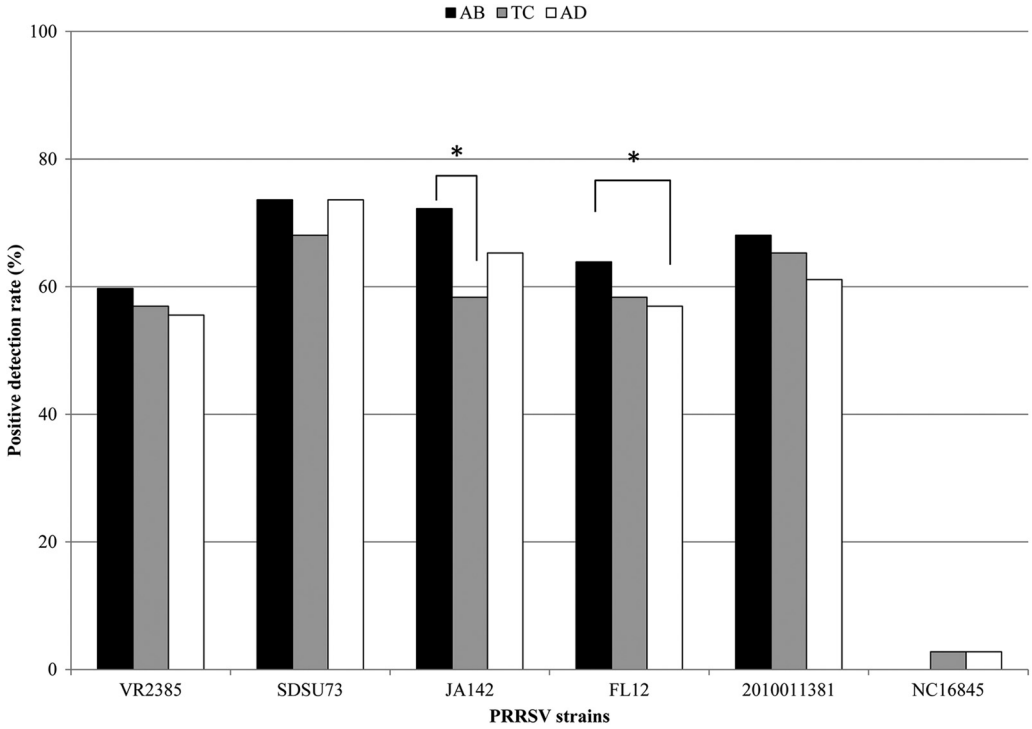

FIG 2 Cumulative detection rates of PRRSV RNA in serum, blood swab, oral fluids and semen of experimentally infected boars with PRRSV strains VR2385, SDSU73, JA142, 2010011381, and FL12 on days postinfection 1 to 21 by three real-time RT-PCR tests (AB, TC, and AD). Asterisks indicate differences between assays (McNemar's test, $P < 0.05$).

Detection of PRRSV RNA and sensitivity of the three commercial real-time assays for heterologous PRRSV isolates. Considering all five PRRSV strains tested, AB detected 67.2% of positive samples (242/360), followed by AD (62.5%; 225/360) and TC (61.7%; 221/360). The AB assay had the highest PRRSV RNA detection rate among the three assays ($P < 0.01$). There was no difference between detection rates of the AD and the TC assays ($P = 0.72$). The results for each strain are summarized in Fig. 2. The AB test had a higher detection rate than TC for group 3 (51/72, or 70.8%, versus 42/72, or 58.3%; $P < 0.01$) and a higher detection rate than AD for group 4 (46/72, or 63.9%, versus 40/72, or 55.6%; $P = 0.03$).

Effects of pooling. To evaluate the effect of the size of sample pools on sensitivity of the RT-PCR, pools of 2, 3, 5, or 10 serum or blood swab samples which contained only one low-, moderate-, or high-positive sample per pool were tested. There was no difference in detection rates among strains or assays. The results of strains and assays were combined and are summarized in Fig. 3. For high-positive samples (C_T of ≤29.9), pooling of 2, 3, 5, or 10 serum or blood swab samples did not reduce the probability of identifying a single positive sample among them. For moderate-positive samples (C_T between 30.0 and 34.9), pools of five serum or blood swab samples still resulted in a positive signal (C_T of <37). One out of five serum sample pools of 10 samples was positive. Pools of 10 samples produced a C_T of >37 for 2/5 strains using blood swab samples and for 1/5 strains using serum samples. When samples were low positive (C_T value between 35.0 and 37.0), pools of two samples were positive in 4/5 strains of serum and blood swab samples. Larger pool sizes gave negative results with low-positive samples although 4/5 and 3/5 serum samples produced C_Ts of >37 in pools of three and five, respectively. Among blood swab samples, 2/5 and 1/5 produced C_Ts of >37 in pools of three and five samples, respectively.

Negative oral fluids and serum samples from the field. To evaluate the prevalence of false-positive results between assays, oral fluid and serum samples were obtained from farms considered PRRSV negative on the basis of regular PRRSV-negative serological test results. All of the 200 serum samples and the 200 oral fluid samples tested negative with the AB and the TC assays. Three of 200 (1.5%) oral fluid samples and 2 of 200 (1.0%) serum samples were positive with C_Ts ranging from 30.8 to 36.7 using the AD assay. All positive results were retested by conventional RT-PCR, and two of three oral fluid samples and one of two serum samples were confirmed PRRSV positive for type 2 (two oral fluid samples) or type 1 (one serum sample) PRRSV. Sequencing of the amplified products was attempted to confirm the presence of PRRSV; however, none of the three "positive samples" yielded a sequence consistent with PRRSV, indicative of nonspecific RT-PCR amplification. Subsequent submissions from the herds with positive results were monitored, with consistent PRRSV-negative ELISA results for at least 2 months after the AD positive results (data not shown).

Turnaround time. Semen samples required additional centrifugation and resuspension steps prior to nucleic acid extraction. The extraction protocol for semen samples and oral fluids required an additional lysis step before the automated extraction on the KingFisher platform compared to serum samples and blood swabs. Considering the amount of time from the start of sample processing until the end of nucleic acid extraction, serum and blood swab samples required 1 h. For processing, the time was increased by 25 min for oral fluids and by 55 min for semen sam-

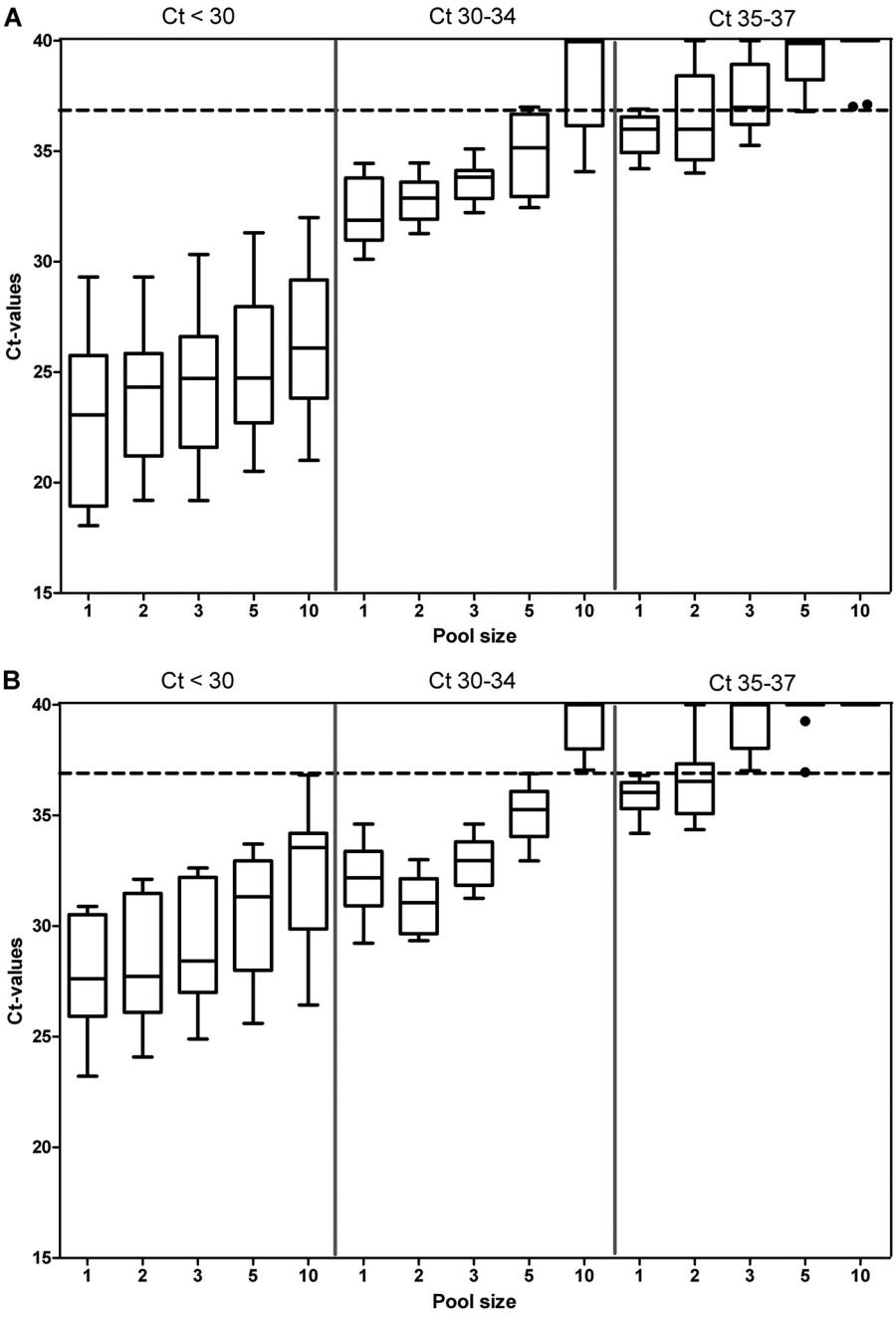

FIG 3 Effects of sample pool size on detection of a single positive sample in the pool of serum (A) and blood swab (B) samples by RT-PCR. A positive sample was diluted in negative-control samples so as to simulate pool sizes of 1, 2, 3, 5, or 10 samples. Three positive ranges were used for each pool representing high-positive (cycle threshold [C_T] of < 29.5), moderate-positive (C_T of 30 to 34.5), and low-positive (C_T of 35 to 37) samples. The horizontal line at C_T 37 represents the cutoff for negative samples (C_T of >37). The box-and-whiskers plots show cumulative results of one tested sample of each strain (VR2385, SDSU73, JA142, 2010011381, and FL12) tested by AB, TC, and AD assays.

ples, totaling 1 h 25 min for oral fluids and 1 h 55 min for semen samples. Regarding the time to complete the PCR, the AB assay required 1 h 30 min, and the TC and the AD assays required an additional 51 min in the run time length, totaling 2 h 21 min.

DISCUSSION

For the detection of heterologous strains of PRRSV RNA, diagnosticians can choose from different commercially available sets of reagents. Veterinarians also vary in what type of sample (blood, serum, oral fluid, and semen) they submit to the diagnostic laboratory. Accurate PRRSV diagnosis is extremely important, as false-positive or false-negative results can cause substantial risks, expense, and economic losses. Therefore, in this study, a head-to-head comparison of three commercially available RT-PCR assays was performed to compare early detection of PRRSV RNA in boars infected experimentally with genetically different strains of PRRSV.

Recently, increased rates of false-negative results were reported in several PRRSV RT-PCR assays commonly used in veterinary diagnostic laboratories in Europe and North America, and combined use of different assays or PCR tests has been recommended to improve the diagnostic success for PRRSV (3–5). In the present study, all of the PRRSV strains used in the study were detected by the three assays. Whereas the AB test had the overall highest detection rate among the assays, the TC and AD tests had overall similar detection rates across all strains.

Negative field samples rather than a group of sham-inoculated boars were included in this study to determine the specificity of the PCR assays. This strategy allowed access to a larger number of independent samples as opposed to a few dependent samples from sham-inoculated boars and was considered a better approach to investigate false-positive frequencies of the PCR assays utilized. To further rule out possible room-to-room contaminations, sequencing of the PRRSVs present in each room and group was conducted at termination of the study. With respect to the specificity among assays using field samples from historically PRRSV ELISA-negative herds, the AD assay resulted in 1.0% (serum samples) to 1.5% (oral fluids) positive results which could not be confirmed by sequencing or further serological testing. Furthermore, the TC and AD assays also generated false-positive results in experimental group 6. False-positive results can have a great economic impact for the producers and industry due to the immediate hold of all semen samples from the stud, retesting of all the boars, and likely culling of suspect positive boars.

The most common samples used for PRRSV monitoring include serum, blood swabs, oral fluids, and semen. Under the conditions of the present study, serum and blood swabs had the best overall performance based on the detection rates during the acute phase of infection, the nearly complete agreement between the three diagnostic assays tested, and the turnaround time. This result is in agreement with previous studies which compared PRRSV RNA detection in serum, blood swabs, and semen (7, 9). The method used in this study to collect blood swabs and serum samples by accessing the saphena vein did not require any animal restraint, and animals could be easily sampled during ejaculation.

In contrast to previous studies, in the present study a high percentage of the boars infected experimentally with PRRSV were PRRSV RNA positive in semen starting at day 1 p.i. To our knowledge the strains used in this study have not been used in other published studies investigating PRRSV shedding in semen, which may partially explain the current findings. Reports describing the onset of PRRSV shedding in semen have varied from 48 to 120 h postinfection while initial PRRSV RNA detection in serum has been consistent (8, 9, 20–23). In addition to PRRSV strain variations, differences in the inoculation method and viral dose used, as well as the RNA extraction method and RT-PCR assay used, could account for the variability in results between studies. Alternatively, the semen samples could have been contaminated with PRRSV during the semen collection and processing. In theory, contamination during sample collection in the animal rooms could have occurred for all sample types except serum samples, as those were collected in a closed tube, thereby reducing the risk of environmental contamination. To reduce the risk of sample contamination within the animal room, collection of oral fluids was conducted first, followed by collection of the other three sample types. In addition, gloves were changed between animals, and one technician collected semen while another technician collected serum first and then blood swabs. After collection, all samples were stored separately by sample type and transported to the lab. The chances of sample contamination during sample processing in the lab were minimized by processing all samples on the day of collection, and the samples were separated chronologically and by technician. All sample manipulations for semen and serum were done in a certified biosafety cabinet, and gloves were changed between groups. Gloves were routinely changed between all oral fluid samples. Further controls for this particular step such as using known negative samples from noninfected boars would have been ideal but were not available due to lack of a negative-control group; however, except for one serum sample and one blood swab collected on day 7 p.i., all samples from the noninfected group 6, which were processed with all other samples, were negative on all three PCR assays tested, further supporting a lack of cross-contamination during this step. Also, the extra steps required to prepare the semen samples for RNA extraction, in addition to the extra steps necessary during the RNA extraction required for semen samples, could have increased the chance of sample contamination during processing. However, none of the negative controls used during the sample processing and PCR plate setup were positive.

Recently, oral fluid sample submissions for surveillance and diagnosis have increased due to the ease of this collection method and cost effectiveness of virus surveillance in pig herds (24). A previous study has shown similar PRRSV RNA detection rates between oral fluids and serum samples of experimentally infected boars (6). However, in this study, oral fluids had the lowest overall RT-PCR detection rates, the highest disagreement between assays, and an increased turnaround time compared with blood swabs and serum samples. In contrast to the previous study, in which PRRSV detection increased from 10% (7/69) on day 1 p.i. up to 100% (67/67) on day 4 p.i., the detection rate on day 1 p.i. in the present study was 60% (9/15), with the lowest detection on day 7 p.i. (5/15). As both studies used the same extraction methods and the AB reagents, the difference in results may be due to differences between inoculation methods and strains used. Another explanation for the disparity between studies and a decrease in detection rates on day 7 p.i. could be differences in sample storage and processing.

Oral fluid matrix is known to contain inhibitors that can cause reaction failure or can reduce analytical sensitivity (25). Although an optimized extraction protocol to overcome this was used in addition to the use of a double amount of the recommended PCR enzymes for the AB test (25), due to the limited amount of this reagent in the TC and the AD reagents, this strategy could not be used for these two assays. This could explain the clear difference in detection rates between the assays used in this study, in which the AB assay presented the highest detection rates on oral fluids using the optimized PCR protocol while the detection rates were lowest for the TC test. This difference was not noticed in the other sample types tested, indicating that PRRSV PCR optimization for oral fluids may also be required for the other two assays.

Sample pooling can result in major savings, particularly of consumables and labor, thereby reducing cost. Pooling of blood swabs and serum samples is now used routinely for boar stud monitoring purposes. Results from this study further support previous findings that pooling serum samples is more effective than pooling blood swabs (9). A pool size of 10 for both serum and blood swab samples substantially increased the number of false-negative

results for moderate- and low-positive samples and should be avoided. There was also a significant decrease in sensitivity when low-positive samples were tested in pools of three and five. This is consistent with another study that determined that up to 14% of positive samples would be missed if pools of five serum or blood swabs were used during the first 5 days of PRRSV infection (9). Due to the dilution effect, it has been suggested that increasing the cutoff value for preliminary evaluation of pools should be used, and retesting pooled samples with C_T values close to cutoff values as single samples should be conducted to avoid false-negative results (11). Under the conditions of the present study, the detection rate in low-positive samples would have increased substantially, and the detection in serum pools of five samples would have increased from no positive samples to 3/5 positive results if a C_T of >37 were considered. As the use of a higher cutoff would likely decrease the specificity of the test, thus potentially increasing the number of false-positive results, this strategy should be further optimized by testing serial sensitivities and specificities at various C_T cutoff points before being applied. Therefore, care must be taken when using pooled samples for testing.

The three boars in group 6 remained negative for anti-PRRSV antibodies throughout the study and were considered noninfected. The reason for this might be technical failure in infecting the boars, a lack of infectious virus in the inoculum used, a single inoculation time, or an insufficient amount of infectious virus. Previous studies dealing with PRRSV experimental infection have shown differences regarding the infectious doses needed to infect animals. For the strain VR-2332, the infectious doses estimated to infect 60% and 90% of 2-week-old pigs by an intranasal inoculation route were estimated to be $10^{4.4}$ and 10^6 $TCID_{50}$s, respectively (26). Other studies using intranasal inoculation with 10^4 $TCID_{50}$s of PRRSV isolate MN 30-100 in boars reported a successful infection rate of 62% (18/19) or 73% (29/40) of the boars, respectively (9, 22). All samples originated from the boars used in this study were PCR negative, with the exception of one serum and one blood swab sample from the same boar on day 7 p.i., which produced a positive signal by both the TC and the AD assays. However, the sequencing of this fragment revealed an unspecific amplification.

In summary, the detection rate for PRRSV RNA varied depending on the sample type and virus isolate used under the conditions of this study. Serum and blood swabs had the best overall performance, with the highest detection rates and agreement between kits. The AB reagents had the highest detection rate across the PRRSV isolates used in this study. Testing pooled samples can compromise the detection rates when low-positive samples are included.

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REFERENCES

1. **Corzo CA, Mondaca E, Wayne S, Torremorell M, Dee S, Davies P, Morrison RB.** 2010. Control and elimination of porcine reproductive and respiratory syndrome virus. Virus Res. **154**:185–192.
2. **Shi M, Lam TT, Hon CC, Hui RK, Faaberg KS, Wennblom T, Murtaugh MP, Stadejek T, Leung FC.** 2010. Molecular epidemiology of PRRSV: a phylogenic perspective. Virus Res. **154**:7–17.
3. **Harmon KM, Abate SA, Chriswell AJ, Strait WAE.** 2012. Comparison of two commercial real-time reverse transcriptase polymerase chain reaction assays for porcine reproductive and respiratory syndrome virus. Swine Health Prod. **20**:184–188.
4. **Toplak I, Rithtaric D, Hostnik J, Grom J, Stukelj M, Valencak Z.** 2012. Identification of a genetically diverse sequence of porcine reproductive and respiratory syndrome virus in Slovenia and the impact on the sensitivity of four molecular tests. J. Virol. Methods **179**:51–56.
5. **Wernike K, Bonilauri P, Dauber M, Errington J, LeBlanc N, Revilla-Fernández S, Hjulsager C, Isaksson M, Stadejek T, Beer M, Hoffmann B.** 2012. Porcine reproductive and respiratory syndrome virus. Interlaboratory ring trial to evaluate real-time reverse transcription polymerase chain reaction detection method. J. Vet. Diagn. Invest. **24**:855–866.
6. **Kittawornrat A, Prickett J, Chittick W, Wang C, Engle M, Johnson J, Patnayak D, Schwartz T, Whitney D, Olsen C, Schwartz K, Zimmerman J.** 2010. Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? Virus Res. **154**:170–176.
7. **Reicks DL, Muñoz-Zanzi C, Rossow K.** 2006. Sampling of adult boars during early infection with porcine reproductive and respiratory syndrome virus for testing by polymerase chain reaction using new blood collection technique (blood swab method). Swine Health Prod. **14**:258–264.
8. **Christopher-Hennings J, Holler LD, Benfield DA, Nelson EA.** 2001. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J. Vet. Diagn. Invest. **13**:133–142.
9. **Rovira A, Clement T, Christopher-Hennings J, Thompson B, Engle M, Reicks D, Muñoz-Zanzi C.** 2007. Evaluation of the sensitivity of reverse-transcription polymerase chain reaction to detect porcine reproductive and respiratory syndrome virus on individual and pooled samples from boars. J. Vet. Diagn. Invest. **19**:502–509.
10. **Johnson W, Roof M, Vaughn E, Christopher-Hennings J, Johnson CR, Murtaugh MP.** 2004. Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. Vet. Immunol. Immunopathol. **102**:233–247.
11. **Fereidouni SA, Harder TC, Gaidet N, Ziller M, Hoffmann B, Hammoumi S, Globig A, Starick E.** 2012. Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. J. Virol. Methods **186**:119–125.
12. **Ladman BS, Spackman E, Gelb J, Jr.** 2012. Comparison of pooling 11 or 5 oropharyngeal swabbings for detecting avian influenza virus by real-time reverse transcription-PCR in broiler chickens. Avian Dis. **56**:227–229.
13. **Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MA, Andrews JJ, Rathje JA.** 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. **32**:648–660.
14. **Mengeling WL, Lager KM, Vorwald AC.** 1998. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. Am. J. Vet. Res. **59**:1540–1544.
15. **Gauger P, Faaberg KS, Guo B, Kappes MA, Opriessnig T.** 2012. Genetic and phenotypic characterization of a 2006 United States porcine reproductive and respiratory virus isolate associated with high morbidity and mortality in the field. Virus Res. **163**:98–107.
16. **Prickett J, Simer R, Christopher-Hennings J, Yoon KJ, Evans RB, Zimmerman JJ.** 2008. Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. J. Vet. Diagn. Invest. **20**:156–163.
17. **Truong HM, Lu Z, Kutish GF, Galeota J, Osorio FA, Pattnaik AK.** 2004. A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. Virology **325**:308–319.
18. **Halbur P, Bush E.** 1997. Update on abortion storms and sow mortality. Swine Health Prod. **5**:73.
19. **Ni YY, Huang YW, Cao D, Opriessnig T, Meng XJ.** 2011. Establishment

of a DNA-launched infectious clone for a highly pneumovirulent strain of type 2 porcine reproductive and respiratory syndrome virus: identification and in vitro and in vivo characterization of a large spontaneous deletion in the nsp2 region. Virus Res. **160**:264–273.
20. **Christopher-Hennings J, Nelson EA, Nelson JK, Benfield DA.** 1997. Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. Am. J. Vet. Res. **58**:40–45.
21. **Christopher-Hennings J, Dammen M, Nelson E, Rowland R, Oberst R.** 2006. Comparison of RNA extraction methods for the detection of porcine reproductive and respiratory syndrome virus from boar semen. J. Virol. Methods **136**:248–253.
22. **Reicks DL, Muñoz-Zanzi C, Mengeling W, Christopher-Hennings J, Lager K, Polson D, Dee S, Rossow K.** 2006. Detection of porcine reproductive and respiratory syndrome virus in semen and serum of boars during the first six days after inoculation. Swine Health Prod. **14**:35–41.
23. **Wasilk A, Callahan JD, Christopher-Hennings J, Gay TA, Fang Y, Dammen M, Reos ME, Torremorell M, Polson D, Mellencamp M, Nelson E, Nelson WM.** 2004. Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum samples by real-time PCR. J. Clin. Microbiol. **42**:4453–4461.
24. **Ramirez A, Wang C, Prickett JR, Pogranichniy R, Yoon KJ, Main R, Johnson JK, Rademacher C, Hoogland M, Hoffmann P, Kurtz A, Kurtz E, Zimmerman J.** 2012. Efficient surveillance of pig populations using oral fluids. Prev. Vet. Med. **104**:292–300.
25. **Chittick WA, Stensland WR, Prickett JR, Strait EL, Harmon K, Yoon KJ, Wang C, Zimmerman JJ.** 2011. Comparison of RNA extraction and RT-PCR methods for the detection of porcine reproductive and respiratory syndrome virus (PRRSV) in porcine oral fluid specimens. J. Vet. Diagn. Invest. **23**:248–253.
26. **Hermann JR, Munoz-Zanzi CA, Roof MB, Burkhart K, Zimmerman JJ.** 2005. Probability of porcine reproductive and respiratory syndrome (PRRS) virus infection as a function of exposure route and dose. Vet. Microbiol. **110**:7–16.
27. **Landis JR, Koch GG.** 1977. The measurement of observer agreement for categorical data. Biometrics **33**:159–174.