Journal of Clinical Microbiology, Oct. 2004, p. 4453–4461 Vol. 42, No. 10
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.10.4453–4461.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

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

A. Wasilk,[1] J. D. Callahan,[2] J. Christopher-Hennings,[1]* T. A. Gay,[2] Y. Fang,[1] M. Dammen,[1] M. E. Reos,[2] M. Torremorell,[3] D. Polson,[4] M. Mellencamp,[5] E. Nelson,[1] and W. M. Nelson[2]

Department of Veterinary Science, South Dakota State University, Brookings, South Dakota[1]; Tetracore, Inc., Gaithersburg, Maryland[2]; PIC USA, Franklin, Kentucky[3]; Boehringer Ingelheim Vetmedica, Ames, Iowa[4]; and Sygen International, Berkeley, California[5]

Received 28 January 2004/Returned for modification 3 June 2004/Accepted 20 June 2004

Transmission of porcine reproductive and respiratory syndrome virus (PRRSV) via boar semen has been documented. Since semen is widely disseminated for artificial insemination and the virus can cause significant health and economic consequences, it is essential to have well-validated, rapid diagnostic techniques to detect and quantitate the virus for diagnostic and research purposes. Previously, boar semen was tested by a nested PCR (nPCR) assay which was compared to the "gold standard" swine bioassay. A correlation of 94% was observed, indicating that, most of the time, PCR detected infectious virus. Subsequently, a real-time PCR targeting the 3′ untranslated region of the PRRSV genome was compared with nPCR by testing 413 serum and semen samples from PRRSV-inoculated and control boars. There was 95% agreement between the results of the two tests, with the majority of samples with discordant results containing virus at the lower range of detection by the assays. The virus in all samples was quantitated by using a standard curve obtained by serial dilution of an in vitro transcript. By using the in vitro transcript, the lower limit of sensitivity was observed to be approximately 33 copies/ml. Reactivity with a panel of more than 100 PRRSV isolates from various geographical regions in the United States was also documented. No reactivity with nine nonrelated swine viruses was noted. A real-time PCR was also developed for the detection of the European Lelystad virus and the European-like PRRSV now found in the United States. In six of six PRRSV-inoculated boars, peak levels of viremia occurred at 5 days postinoculation (DPI) and were most consistently detectable throughout 22 DPI. In five of six boars, PRRSV was shed in semen for 0 to 2 days during the first 10 DPI; however, one of six boars shed the virus in semen through 32 DPI. Therefore, in general, the concentration and duration of PRRSV shedding in semen did not correlate with the quantity or duration of virus in serum. These differences warrant further studies into the factors that prevent viral replication in the reproductive tract.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded RNA virus belonging to the order *Nidovirales*, family *Arteriviridae*, along with lactate dehydrogenase-elevating virus of mice, equine arteritis virus, and simian hemorrhagic fever virus (4, 13). PRRSV causes significant respiratory disease in nursery and growing-finishing pigs and late-term abortions in sows (29, 46). Since the etiologic agent was identified in 1991, multiple methods of virus dissemination have been discovered, including transmission via semen (2, 12, 18, 30, 47, 50). Transmission of PRRSV through the semen of infected boars may not always occur, but due to the widespread use of artificial insemination in the modern swine industry, dissemination of PRRSV via semen constitutes a major risk for swine herds. Therefore, it is important to provide diagnostic testing to ensure a PRRSV-free semen supply (41). Interestingly, the duration of PRRSV shedding in semen is quite variable among individual boars, ranging from as short as 4 days postinoculation (DPI) to as long as 92 DPI (5–11). The viral loads in semen and serum samples from boars, which may have an effect on transmission, have not been extensively studied quantitatively. Intermittent shedding of PRRSV in semen may also occur and may not necessarily correlate with viremia or serological status (6). This necessitates the use of excellent biosecurity measures, and for many boar studs, PCR testing of individual or pooled semen samples that will be used for sow inseminations is done. Since virus isolation (VI) from boar semen samples is insensitive due to the cytotoxicity of semen, PCR, particularly a nested PCR (nPCR) with primers derived from open reading frame (ORF) 7, has been extensively used for PRRSV detection in semen samples (5–11). This method has been well validated and compared to a swine bioassay which detects infectious virus by injection of 13 of 15 ml of semen intraperitoneally into 3-week-old PRRSV-naïve piglets (5, 28, 42). Piglet serological status is then monitored to determine whether seroconversion from the injected PRRSV-contaminated semen occurred. Previously, it was found that there was a 94% correlation between the results of nPCR and those of swine bioassay in studies with semen samples, indicating that, in the majority of cases, if the nPCR result was positive, then it was detecting infectious virus within the semen sample (5). Since nPCR is quite labor-intensive, an automated

* Corresponding author. Mailing address: Animal Disease Research and Diagnostic Laboratory, South Dakota State University, N. Campus Dr., Box 2175, Brookings, SD 57007-1396. Phone: (605) 688-5171. Fax: (605) 688-6003. E-mail: Jane_Christopher-Hennings@sdstate.edu.

Downloaded from jcm.asm.org by on March 4, 2008

real-time PCR was developed for rapid, user-friendly detection and relative quantitative analysis. Since the test is also commercially available, the standardization of testing between laboratories may be possible. The purpose of this study was to develop and validate this real-time PCR by comparison with nPCR and to document inter- and intra-assay variabilities, sensitivity, and specificity. In addition, since quantitation would be useful for pathogenesis, treatment, and vaccine efficacy studies, a standard curve was developed by using in vitro transcription and measurements of the viral loads in serum and semen samples from experimentally infected boars were obtained.

MATERIALS AND METHODS

Experimental animals. Nine boars (eight of the Landrace breed and one of the Duroc breed; age range, 6 to 9 months) were obtained from a PRRSV-negative commercial breeding herd. The boars were PRRSV and pseudorabies virus negative and were confirmed by serology and PCR to be PRRSV negative prior to delivery and again on arrival. Six principal boars and three control boars were housed in individual isolation rooms, with each group housed in separate buildings. Boars were acclimated for 2 weeks prior to PRRSV inoculation. Semen was collected during the first week to familiarize the boars with the collection procedures. Three collections of serum and semen samples were obtained during the second week for preinoculation baseline PCR, enzyme-linked immunosorbent assay (ELISA), and serum neutralization (SN) analysis.

Viruses. A virulent, low-passage (passage 6) isolate (isolate SD-23983) of PRRSV was propagated on MARC-145 cells (23) and used as the inoculum. The cells were frozen and thawed twice to release the virus. Cell debris was removed by low-speed centrifugation at 500 × *g* for 5 min. The supernatant containing the virus was stored as 2-ml aliquots and kept frozen at −80°C. The amount of virus was determined by titration on MARC-145 cells in a microtiter assay and had an infectivity titer of 10^6 50% tissue culture infective doses ($TCID_{50}$s)/ml. To determine the sensitivity of the real-time PCR for Lelystad and European-like PRRSVs, a stock Lelystad virus was serially diluted 10-fold, and nPCR and real-time PCR were performed in parallel with the identical dilutions. Twenty-seven serum samples were also submitted to the Animal Disease Research and Diagnostic Laboratory (ADRDL) at South Dakota State University from various states within the United States, and the viruses in these samples were identified as European-like PRRSV by real-time PCR and were confirmed by genomic sequencing of ORF 5.

Experimental inoculation. Each naris of the six principal boars was inoculated with 1 ml of PRRSV isolate SD-23983 (10^6 $TCID_{50}$s/ml). This viral isolate and dose were previously used for boar inoculations (11). Each naris of the control boars were inoculated with 1 ml of a mock-infected cell supernatant which was PRRSV negative, as determined by VI and PCR.

Blood collection. Twelve milliliters of blood was collected from the jugular vein and transferred to a serum separator Vacutainer tube for centrifugation at 950 × *g* for 10 min. Quantitative, real-time PCR and nPCR analyses of the serum samples were performed on the day of sample collection.

Semen collection. Whole semen was collected three times per week for 4 weeks, two times per week for 2 weeks, and then once per week for 6 weeks by the gloved-hand technique (24). Semen samples were collected in insulated containers covered with gauze to remove the gel fraction from the ejaculate.

Semen preparation. Ten milliliters of whole semen was centrifuged at 600 × *g* for 20 min. The seminal plasma was removed, and the pellet was resuspended in a volume of sterile phosphate-buffered saline equal to the volume of the pellet. Quantitative PCR and nPCR analyses of the semen samples were performed on the day of sample collection.

Comparison of phenol-chloroform method and Qiagen RNeasy spin column protocols for extraction of RNA from semen samples. A previously used guanidinium isothiocyanate (GITC) buffer (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7], 0.5% *N*-lauryl sarcosine)–phenol-chloroform extraction (GITC–phenol-chloroform) method (5–11) and a spin column technique (RNeasy; Qiagen, Valencia, Calif.) were compared. Proteinase K-HIRT (PK-H) buffer (200 μg of proteinase K per ml, 20 mM Tris, 20 mM EDTA [pH 8.0], 2% sodium dodecyl sulfate) was also used prior to the Qiagen spin column method to solubilize the semen (25, 35). Ten semen samples were obtained from a boar stud which had become PRRSV infected and were evaluated by the two methods. In addition, these samples were evaluated with the PK-H buffer along with GITC buffer or PK-H buffer along with the Qiagen RLT buffer supplied with the RNeasy kit and by subsequent processing through the Qiagen RNeasy spin column. Fourteen additional samples from the present experimental study were used, and semen extraction was performed by the GITC–phenol-chloroform method or by the method with PK-H buffer-GITC buffer and the Qiagen RNeasy kit.

Extraction of RNA from semen samples. To obtain viral RNA, 500 μl of the seminal cell fraction was added to 500 μl of PK-H buffer, and the components were mixed thoroughly and heated on a 37°C heat block for 10 min. The sample was centrifuged at 16,000 × *g* for 30 s. Five hundred microliters of supernatant was added to 500 μl of GITC lysis buffer, which replaced the RLT buffer supplied with the RNeasy kit. The GITC lysis buffer and sample mixture were added to 500 μl of 70% ethanol, and the components were mixed and transferred to the Qiagen RNeasy spin column. The remaining extraction procedure was performed as described in the instructions accompanying the RNeasy kit.

Extraction of RNA from serum. The QIAamp Viral RNA Mini kit was used to obtain viral RNA from the serum samples, as described in the instructions provided with the kit. A 140-μl volume of serum was used for the extraction.

nPCR. A reverse transcriptase (RT) nPCR assay developed previously (5) was used to detect PRRSV RNA in the semen and serum samples. Outer and nested primers were derived from ORF 7 for the detection of U.S. PRRSV and ORF 1b for the detection of the European Lelystad virus (5).

Real-time PCR. A commercially available real-time, single-tube RT-PCR assay for the detection of U.S., Lelystad, and European-like PRRSVs was provided by Tetracore Inc. (Gaithersburg, Md.) and was used to detect PRRSV RNA. A minor groove binding 5′ nuclease probe and primers specific for the 3′ untranslated region (UTR) of the PRRSV genome were designed by alignment of sequences from GenBank and were based on conserved areas of the 3′ UTR-specific primer and probe region. The PRRSV RNA was transcribed in a single tube by using a 25-μl reaction volume consisting of Tetracore U.S. PRRSV master mixture (18.9 μl of the master mixture, 2 μl of enzyme mixture 1, 0.1 μl of enzyme mixture 2) and 4 μl of extracted RNA. The reaction tubes were loaded into a Smart Cycler II block (Cepheid, Sunnyvale, Calif.), and software settings for fluorescence detection were set for automatic calculation of the baseline with the background subtraction turned on. The thermal cycler program for the U.S. PRRSV real-time assay consisted of 52°C for 1,800 s; 95°C for 900 s; and 45 cycles at 94°C for 30 s, 61°C for 60 s, and 72°C for 60 s. For the Lelystad and European-like PRRSV assay, thermal cycling times consisted of 60°C for 1,200 s, 95°C for 15 s, and 45 cycles at 95°C for 3 s and 60°C for 30 s. A PCR was considered positive if the cycle threshold (C_t) level was obtained at ≤45 cycles.

Normalization. To determine whether there was extraction inefficiency and/or RT-PCR inhibition from serum or semen samples, prior to the extraction procedure three dilutions of virus from cell culture were spiked into serum and semen samples from PRRSV-inoculated boars obtained preinoculation. A total of 10^6 $TCID_{50}$s of virus per ml was spiked into the sample at dilutions of 10^{-1}, 10^{-3}, and 10^{-5} (10^5, 10^3, and 10^1 $TCID_{50}$s/ml, respectively), and the C_t values and the numbers of RNA copies per milliliter were compared to those values in cell culture alone. Extractions of virus from cell culture and spiked samples were performed in triplicate for each dilution and each individual boar sample.

In vitro transcription. A 102-bp PCR fragment specific for the 5′ nuclease probe target area of U.S. isolate SD-23983 was purified and used as a template for in vitro transcription. The PCR product was purified with a Qiagen Qiaquick PCR purification kit according to the instructions of the manufacturer and ligated into the pGEM-T vector by using pGEM-T Easy Vector system (Promega, Madison, Wis.). The ligation product was transformed into TOPO 10F cells, and plasmid DNA was extracted by using a Qiagen Mini Plasmid Prep kit. After linearization, the in vitro transcription reaction was performed with an SP6 mMESSAGE mMACHINE Transcription kit (Ambion, Austin, Tex.), and the newly transcribed RNA was recovered with NucAway spin columns (Ambion). The RNA concentration was determined by measurement of the optical density by spectrophotometry.

Standard curve. Known amounts of the serially diluted RNA transcript obtained in vitro (10^{-1} through 10^8 copies/μl) were used to generate a standard curve. Four microliters of each dilution was added to the PCR mixtures; the PCRs were performed three to six times each day over 3 days; and the mean value, the coefficient of variation, and minimum level of detection of the in vitro transcript were determined (Fig. 1). The concentrations in the unknown samples (in numbers of RNA copies per milliliter) were determined by linear extrapolation of the C_t values plotted against the known concentration of the 3′ UTR transcription product.

Virus isolation from sera. One hundred microliters of each serum sample was used to prepare 10-fold serial dilutions (10^{-1} to 10^{-6}) in minimal essential medium. Two hundred microliters of each dilution was added to triplicate wells of a microtiter plate containing confluent monolayers of MARC-145 cells. The plates were incubated for 48 h in a humidified incubator at 37°C with 5% CO_2.

Downloaded from jcm.asm.org by on March 4, 2008

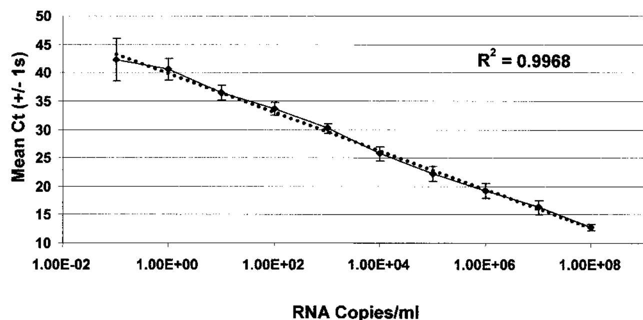

FIG. 1. Standard curve of number of RNA copies per milliliter (dashed line) and 12 replicates of each concentration (3 to 6 replicates per day for 3 days) (solid line) versus the mean C_t ($y = -0.31x + 11.441$). Bars indicate standard errors.

The presence of virus was confirmed by a direct fluorescent-antibody technique with monoclonal antibody SDOW 17 (27). For samples that were PRRSV positive by VI, an additional titration was performed in triplicate by using a serial twofold dilution to obtain more accurate end points for comparisons with quantitative PCR (Fig. 2).

SN analysis. SN analysis was performed as described previously (48). Titers ≥1:4 were recorded as positive.

Antibody ELISA. Serological status was monitored by the commercially available 2XR IDEXX HerdChek PRRS ELISA (IDEXX Laboratories, Westbrook, Mass.), and the results were recorded as a sample-to-positive control ratio. Animals with a ratio of ≥0.4 were considered seropositive for PRRSV.

Statistics. The relationship between all PRRSV-positive virus titrations (numbers of $TCID_{50}$s per milliliter) and the numbers of RNA copies per milliliter of serum for PRRSV-inoculated boars during the first 10 days of the study were evaluated by linear regression analysis. A P value <0.05 was considered significant. Unweighted least-squares linear regression (Statistix, version 8.0, 2003; Analytical Software, Inc., Tallahassee, Fla.) was performed. The model used

FIG. 2. Viral load in serum, in $TCID_{50}$s per milliliter, as determined by virus titration (■), and in numbers of copies per milliliter, as determined by quantitative real-time PCR (□). Values are for the first 10 DPI for each PRRSV-inoculated boar.

Downloaded from jcm.asm.org by on March 4, 2008

log-transformed $TCID_{50}$s per milliliter as the dependent variable and log-transformed numbers of RNA copies per milliliter for the positive values as the predictor variable. Data from samples with negative VI results were excluded from the regression analysis. For the initial model, the intercept was nonsignificant ($P = 0.189$), so the model was rerun with the intercept forced through the origin, and the adjusted R^2 value is reported.

RESULTS

Sensitivity, specificity, and inter- and intra-assay variabilities. By using serial cell culture dilutions for the U.S. isolate (isolate SD-23983), the level of detection by the real-time PCR matched that by the nPCR, which has previously been reported to be 10 $TCID_{50}$s/ml (5). The minimum level of the in vitro transcript that was consistently detected by the RT-PCR was 33 RNA copies/ml. This assay did not react with other common swine viruses, which included swine influenza virus, coronavirus, parvovirus, transmissible gastroenteritis virus, circovirus, and pseudorabies virus. Inter- and intra-assay variabilities of 2.7 to 8.8 and 0.1 to 5.7%, respectively (Fig. 1), were observed. By using serum and semen samples from boars experimentally infected with PRRSV, a 95% correlation between nPCR and real-time PCR was observed, and the kappa score was 0.821, which is considered very good agreement (1). The same numbers of samples were real-time PCR positive and nPCR negative and real-time PCR negative and nPCR positive (Table 1). Quantitative measurements indicated that the majority of samples with discordant results contained RNA at levels near the minimum threshold values of the assays. Only 1 serum sample of the 138 serum and semen samples from control boars tested was PRRSV positive by real-time PCR, and this occurred at 3 DPI ($C_t = 40.12$). This sample was PRRSV negative by nPCR, so in this experimental study the specificities of the real-time PCR and nPCR were 99 and 100%, respectively. The experimental study demonstrated limited virus shedding in semen in five of six boars, despite the high viral loads in serum (Table 1; Fig. 3). PRRSV shedding in semen in boar 6 was detected through 32 DPI, and in this boar the viral load was greater in semen than in serum in most cases (Table 1).

The specificity of the real-time PCR assay was further investigated by alignment of the sequences of 36 PRRSV isolates from GenBank to evaluate the homology of the primers and probes used in the real-time PCR assay. In addition, 134 clinical isolates were obtained through the ADRDL at South Dakota State University from samples from various farms over a wide geographical area in the United States. Fifty-two of these samples were PRRSV positive by both real-time PCR and nPCR and had been selected with diverse predicted RFLP cut patterns by genomic sequencing of ORF 5. In addition, 82 isolates were from PRRSV-positive samples submitted to a diagnostic laboratory that had been tested in parallel by real-time PCR and VI, with corresponding results. To date, the ADRDL at South Dakota State University has detected PRRSV in more than 4,440 samples by determining the RFLP cut pattern by ORF 5 sequencing, documenting the broad reactivity of the assay for many diverse PRRSV isolates by continued comparisons of the results with those of VI and the agreement of the results of the two methods.

The sensitivity of the real-time PCR for the detection of Lelystad and European-like PRRSVs also matched that of the nPCR assay when serial dilutions of Lelystad virus were used

TABLE 1. Viral loads in serum and semen samples from PRRSV-inoculated boars

Boar no. and sample	Viral load (no. of RNA copies/ml) on the following DPI:																						
	1	3	5	8	10	12	15	17	19	22	24	26	29	32	36	39	44	51	58	67	72	79	86
1																							
Serum	9×10^{1}	2×10^{4a}	3×10^{4a}	4×10^{4}	5×10^{1}	1×10^{2b}	0	0	0	2×10^{3}	0	4×10^{3}	0	0	2×10^{2b}	0	0	0	0	0	0	0	0
Semen	0	0	0	4×10^{4}	3×10^{4}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2																							
Serum	2×10^{2}	5×10^{4a}	2×10^{5a}	1×10^{5}	4×10^{4a}	7×10^{2}	3×10^{2}	1×10^{3}	2×10^{3}	0	N[c]	0	0	0	0	3×10^{1b}	0	0	0	0	0	0	
Semen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3																							
Serum	2×10^{3a}	8×10^{4a}	2×10^{5a}	3×10^{4a}	8×10^{1}	7×10^{2}	1×10^{2}	6×1^{1b}	5×10^{1}	1×10^{2b}	N	N	6×10^{3}	0	0	0	0	0	0	0	0	0	0
Semen	0	6×10^{6}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4																							
Serum	3×10^{3}	6×10^{4a}	1×10^{5a}	5×10^{3}	5×10^{1b}	6×10^{3}	3×10^{2}	0	N	2×10^{2}	0	0	0	4×10^{2}	9×10^{1b}	0	0	0	0	0	0	8×10^{1b}	0
Semen	0	0	0	6×10^{2}	2×10^{1b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5																							
Serum	2×10^{3a}	8×10^{4a}	9×10^{5a}	4×10^{5}	3×10^{3}	2×10^{4a}	5×10^{2}	2×10^{2}	5×10^{3}	9×10^{2}	N	3×10^{2}	0	0	6×10^{1b}	0	0	0	0	0	0	0	0
Semen	0	0	9×10^{4}	0	9×10^{1}	0	0	N	N	0	0	0	ND[d]	0	0	0	0	0	0	0	0	0	0
6																							
Serum	3×10^{3a}	5×10^{4a}	2×10^{5a}	5×10^{4a}	7×10^{2}	6×10^{1}	N	4×10^{2}	N	6×10^{1b}	N	3×10^{2}	0	N	0	0	0	0	0	0	0	0	0
Semen	0	0	1×10^{6}	1×10^{5}	2×10^{6}	8×10^{5}	3×10^{4}	3×10^{5}	0	2×10^{6}	1×10^{4}	0	6×10^{3}	3×10^{3}	0	0	0	0	0	0	0	0	0

[a] PRRSV was detected by virus isolation.
[b] nPCR negative, real-time PCR positive.
[c] N, nPCR positive, real-time PCR negative.
[d] ND, no sample was collected.

Downloaded from jcm.asm.org by on March 4, 2008

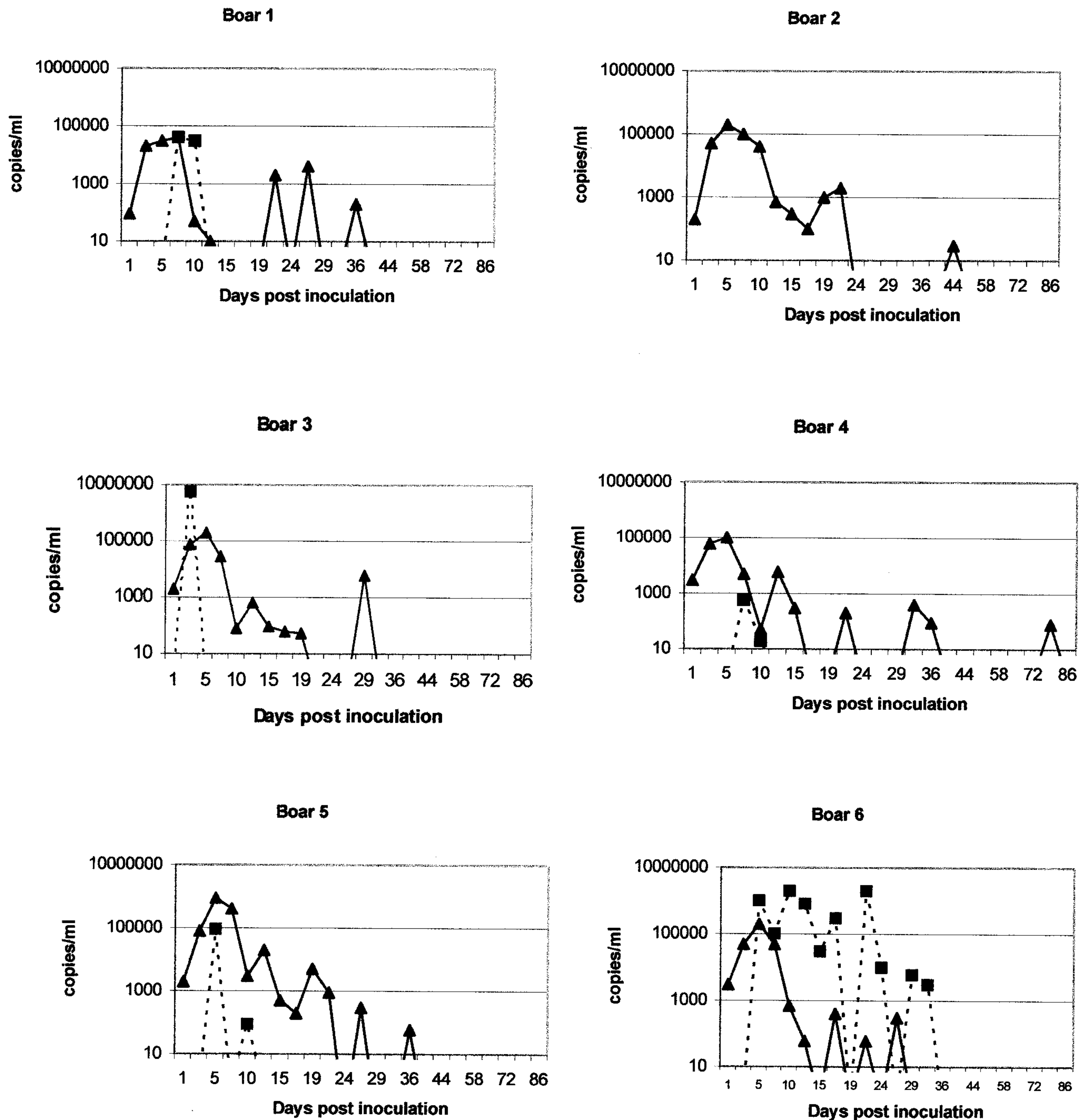

FIG. 3. Viral loads (number of RNA copies per milliliter) and viremia and shedding patterns in PRRSV-inoculated boars. ■, detection of PRRSV, quantitation in semen, and shedding patterns; ◆, PRRSV detection and quantitation in serum.

Downloaded from jcm.asm.org by on March 4, 2008

for comparisons (data not shown). An additional 27 European-like PRRSV isolates detected in the United States were also identified by the Lelystad and European PRRSV real-time assay.

Comparison of phenol-chloroform method and Qiagen RNeasy spin column protocol for extraction of RNA from semen. To determine whether the semen extraction method used in this experimental study was equivalent to the previously used GITC–phenol-chloroform method and to optimize the extraction by using a more user-friendly spin column kit technique, both experimentally and naturally infected semen samples were obtained. Ten of 10 PRRSV-positive semen samples from the naturally infected boar stud were detected by using PK-H buffer, Qiagen RLT buffer, and the Qiagen RNeasy kit, whereas 7 of 10 samples were positive by the GITC–phenol-chloroform method. When RNA was reextracted from nine of the PRRSV-positive samples in parallel by using PK-H buffer, GITC buffer, and the Qiagen RNeasy kit, nine of nine samples were PRRSV positive, whereas six of nine samples were PRRSV positive by using PK-H buffer, Qiagen RLT buffer, and the Qiagen RNeasy kit. When RNA was extracted from 14

semen samples from the experimental study by using PK-H buffer, GITC buffer, and the Qiagen RNeasy kit and by using GITC buffer and phenol-chloroform extraction, the results were identical, except that the assays with PK-H buffer, GITC buffer, and the Qiagen RNeasy kit detected one additional PRRSV-positive sample. Therefore, for the extraction of RNA from semen in this study, PK-H buffer was used along with GITC buffer in place of the Qiagen RLT buffer prior to the use of the RNeasy kit spin columns.

Normalization. No quantitative differences were observed by using cell culture virus dilutions alone and cell culture virus dilutions spiked into serum samples (data not shown). This indicated that there was no inhibition or extraction inefficiency that required normalization for the quantitation of serum viral loads. This lack of inhibition has been documented in other studies that have used serum for virus quantitation (43). The virus concentrations in PRRSV-spiked semen samples were compared with the virus concentrations in cell culture at the three dilutions tested. The mean decrease in the virus concentration in semen for the boars and for the three dilutions of virus was 137 ± 29 RNA copies/ml (standard error). Therefore, the virus concentration in each PRRSV-infected semen sample was multiplied by a factor derived from the mean of the input amount (number of RNA copies per milliliter of cell culture virus only at each dilution) divided by the mean number of RNA copies per milliliter for the virus spiked into preinoculation semen samples from each of the PRRSV-infected boars. To determine whether the decline in the number of RNA copies per milliliter in semen was due to the inefficiency of extraction or RT-PCR inhibition, viral RNA was spiked into previously extracted PRRSV-negative semen samples. No RT-PCR inhibition was noted, as detected by a decrease in the number of copies per milliliter derived from cell culture RNA and cell culture RNA spiked into extracted semen and subsequent RT-PCR (data not shown).

PRRSV detection and concentrations in serum and semen. The viral loads (number of RNA copies per milliliter) in serum and semen samples from PRRSV-inoculated boars and comparison of the results of nPCR and real-time PCR are shown in Table 1 (for 1 through 86 DPI). The patterns of detection of viremia and shedding of PRRSV in semen could be observed by the quantitative real-time PCR (Fig. 3). Linear regression analysis of the data obtained during the first 10 days of the study demonstrated a significant linear relationship between the results of VI (in $TCID_{50}$s per milliliter) and those of real-time PCR (number of RNA copies per milliliter) for serum (Fig. 2) (in the unforced model, adjusted $R^2 = 0.523$ and $P = 0.0004$; in the forced model, adjusted $R^2 = 0.498$ and $P = 0.001$).

Serology. PRRSV antibodies were detected by 8 to 10 DPI in all of the principal boars by ELISA, and the ELISA results remained positive through the remainder of the study (96 DPI). Neutralizing antibodies were detectable by 29 to 32 DPI. Maximum neutralizing antibody titers of 1:16 were detected at 58, 67, and 72 DPI for boar 6, 72 DPI for boar 4, and 67 DPI for boar 5. The remaining three principal boars had maximum serum neutralizing titers of 1:8. None of the control boars seroconverted to PRRSV positivity, as detected by ELISA or SN analysis.

DISCUSSION

This study documents the development and validation of the first commercially available real-time PCR assay for the detection of U.S., Lelystad, and European-like PRRSVs and use of the assay for the quantitation of PRRSV in boar semen and serum samples. The assay is valuable for relative virus quantitation for vaccine, treatment, and pathogenesis studies as well as diagnostic assays. Since the real-time assay is validated for use for the detection of PRRSV in semen from experimentally infected boars, the assay will be useful in determining the viral loads necessary for transmission and studying factors that may reduce or prevent viral replication in the reproductive tract.

PCR assays for the detection of single-stranded RNA viruses are difficult to develop due to the high rate of mutation, which can hinder primer and probe specificities. Since 2001, the ADRDL at South Dakota State University has also detected 27 European-like PRRSV isolates. The nucleotide sequences of the structural and nonstructural ORFs of these isolates are approximately 95.3% similar to those of Lelystad virus, and the nucleotide sequences of the structural ORFs of these isolates are approximately 70% similar (2–7) to those most of typical U.S. strains (32). European-like and U.S. PRRSV strains have also been identified in a single pig, and these were detected simultaneously by both PCR assays with subsequent verification by sequencing (personal observation). It may be useful to develop a multiplex assay that could detect both U.S. and European-like isolates within a single PCR; however, when this was done, some loss of sensitivity was observed (data not shown). The degree of sensitivity is important for the detection of PRRSV, since very low levels have been observed in vivo, particularly in persistently infected pigs, and these low levels may correspond to biologically active virus that VI may not detect (21). Therefore, one of the primary goals of this study was to develop a real-time PCR assay that would have sensitivity equal to that of the nPCR, whose results were previously shown to correspond to those of the "gold standard" swine bioassay and which has been used extensively in experimental studies and for diagnostic evaluations (5–11, 21). Other real-time PCR assays for the detection of PRRSV have been described, but none of those studies validated the quantitative real-time PCR with serum and semen samples from PRRSV-infected boars (3, 17, 37, 44).

Previous studies documenting the validation of semen extraction techniques for the detection of PRRSV in semen have been limited. In fact, most studies have used real-time or traditional PCR without evaluating the diagnostic sensitivity compared with that of a gold standard clinical assay, such as the swine bioassay, and have reported on the sensitivities of the assays only on the basis of studies with serial dilutions of cell culture virus in vitro. The sensitivity of the assay is also dependent on the method of semen processing and the method of extraction of RNA from semen. Seminal plasma has been noted to inhibit PCR; therefore, by the extraction method used in the present study, the seminal plasma was removed by centrifugation and the cell fraction was used for PCR (14, 16). Spermatozoa have also been shown to inhibit PCR; however, this fraction is usually obtained after centrifugation and lysis with a buffer containing dithiothreitol (22, 31, 36). The extraction buffer used in our experiments did not contain a disulfide

Downloaded from jcm.asm.org by on March 4, 2008

disrupting agent, such as dithiothreitol or β-mercaptoethanol, so it is similar to those used in other differential lysis techniques that release only somatic cell DNA and not sperm cell DNA (22, 31, 35, 49). Previously, PRRSV was found most consistently in the non-sperm cell fraction of the semen rather than in whole semen or seminal plasma (5, 6, 10, 36, 40). In vasectomized boars, PRRSV could be found in seminal macrophages by immunohistochemistry, and PRRSV was identified in the cellular fraction in 87% of the positive samples (10). However, the cellular fraction is quite viscous, and there are difficulties with passing this fraction through commercially available extraction spin columns. Since previous studies demonstrated the close correlation between the results of the swine bioassay and the results of nPCR with RNA extraction with GITC buffer–phenol-chloroform, the method with GITC buffer–phenol-chloroform and the Qiagen RNeasy kit was compared with the method with GITC buffer and RLT buffer supplied with the RNeasy kit. Prior to the spin column assay, the semen was solubilized with the HIRT buffer described by Krieger et al. (25). Since the extraction with the commercially available GITC buffer–PK-H buffer performed as well as that with the GITC buffer–phenol-chloroform used previously, comparisons between the present and previous studies can be made.

Previous studies of PRRSV-infected adult boars have demonstrated viremia during the first week after inoculation and have demonstrated that the viremia can be detected for longer periods of time by PCR than by VI (6). This finding was also confirmed in the present study and demonstrates that the PCR assay is more sensitive than VI for the detection of PRRSV-positive animals. Seroconversion is usually detected within 1 to 2 weeks after inoculation, subsequent to the detection of viremia. Variability in the duration of PRRSV shedding in semen has also been observed. Combined, three previous studies demonstrated a range of shedding times from as short as 4 DPI to as long as 92 DPI, with a mean of 35 DPI ($n = 15$ boars) (5, 6, 11). However, shedding was not detected in one boar, and this was the first study in which shedding was not detected. In addition, limited shedding for only 1 to 2 days was observed in four of the six principal boars. It is possible that semen samples were not collected and tested on a day when these boars were shedding virus in their semen. Since the same PRRSV isolate and dose were given to all principal boars simultaneously, it would seem likely that there are host factors affecting the duration of viral shedding. It has been documented with a limited number of pigs that some differences in the duration of shedding may be noted between litters and breeds in their susceptibilities to PRRSV (11, 20). Other than boars 1 and 4, which were from the same litter, no other genetic similarities were noted between the other boars, although all boars except boar 5 were of the Landrace breed. Boar 5 was of the Duroc breed.

Quantitatively, the results of PCR with serum samples demonstrated the highest viral loads in serum (approximately 10^5 copies/ml) at 5 DPI. These then fell 1 to 4 log units by 22 DPI. Similarly, another study also demonstrated maximal levels of PRRSV RNA in the serum of one boar at 2 DPI, with high levels maintained for up to 7 DPI, but these then decreased to $<10^5$ copies/ml (36). Even though viremia could be detected sporadically in all boars after 22 DPI, the majority of samples from these boars demonstrated discordant results; and when the numbers of RNA copies per milliliter were measured, the majority of these real-time PCR-positive, nPCR-negative samples had levels $\leq$90 copies/ml. This would indicate that the discordant results may be due to a low level of virus, and this level should be considered the diagnostically valid level of detection. This intermittency of the detection of viremia has been documented previously at between 78 and 228 days post-farrowing in piglets infected in utero (33). The patterns of shedding in semen were also sometimes intermittent (Fig. 3). Since PRRSV RNA was detectable in the tonsils and lymph nodes from all of the principal boars when they were euthanized at 96 DPI (data not shown), these intermittent patterns may reflect low levels in semen and in serum originating from tissue sites.

A linear relationship between $TCID_{50}$s per milliliter of serum and the numbers of RNA copies per milliliter of serum was observed during the first 10 days of the study, although only about 50% of the variation in virus titration results could be related to the numbers of RNA copies per milliliter (adjusted $R^2 = 0.498$). The model used was intended to evaluate the relationship between two different virus quantification methods in which virus was detected by both methods (VI [$TCID_{50}$ per milliliter] and real-time PCR [numbers of RNA copies per milliliter]). Even though many samples had negative VI results, the relevance of the regression analysis was that across the range of viral loads detectable by both VI and RT-PCR, there was a significant and positive relationship between the two methods of measurement of virus loads. Virus titrations were performed with serum samples and not purified virus. Therefore, more subgenomic viral RNA that may be measured by the PCR may be present, and this would account for the higher number of RNA copies per milliliter than $TCID_{50}$s per milliliter. In addition, before the virus titrations one freeze-thaw cycle was performed with the serum, but this step was not performed before the PCR procedure. This was because all sera were initially identified as PRRSV positive or negative, and then the positive sera were titrated to their end points. Other factors may also affect the number of infectious virions present in the sample, such as variations in culture conditions and the viral isolate and cell type used. However, our results demonstrate a relatively linear association between the amount of infectious virions and the numbers of RNA copies per milliliter detected by quantitative PCR.

To determine whether PCR inhibition or the inefficiency of extraction lowered the level of virus detected in semen, the quantitative results obtained with PRRSV-spiked semen samples were compared with the quantitative results obtained with virus alone after extraction and RT-PCR. For relative quantitation by PCR, some commercial and experimental PCR assays use housekeeping genes to normalize samples to control for sample-to-sample variability. However, these gene concentrations may also vary between individuals, sample types (e.g., tissue and cell types), and disease status (38). It is also important that quantification be based on an internal control that has an amplification efficiency equal to that of the sample (39). This prerequisite can be fulfilled only by using an internal control that would be similar to the actual template being measured. Therefore, we used preinoculation semen samples from principal boars and spiked them with the same PRRSV

Downloaded from jcm.asm.org by on March 4, 2008

isolate found in the naturally infected samples. The number of RNA copies per milliliter from each boar's semen sample was then multiplied by a factor derived from the input number of RNA copies per milliliter (for cell culture virus only) divided by the number of RNA copies per milliliter in the spiked sample (the same cell culture virus concentration spiked into the preinoculation semen sample). This is typically done by the Amplicor HIV-1 Monitor test (Roche Molecular Systems, Branchburg, N.J.), in which a similar formula is used for the normalization of serum and semen samples with in vitro-transcribed RNA (the secondary target), which is differentiated from the primary target in the probe binding region but which uses the same primers (26, 34, 45). The total amount of human immunodeficiency virus (HIV) type 1 (the primary target) in the sample is also multiplied by the input amount of the secondary target sequence alone divided by the amount of secondary target in the spiked sample. It will be useful in the future to develop a commercially available internal control for PRRSV that could be spiked into samples prior to extraction for determination of the extraction inefficiency or PCR inhibition for the individual samples tested.

The concentration of PRRSV in semen typically did not correlate with the levels in serum. This has also been observed with other viruses, such as HIV, in which discordant results were observed between the blood plasma and the seminal plasma compartments in patients infected with HIV and in which intermittent patterns of shedding in semen were also observed (19). This indicates that the quantities of HIV in blood plasma may not accurately predict the risk of transmission via semen (15). This may also be true in boars. For most boars in this study, PRRSV was identified in serum but not semen on the majority of collection days. For others, such as boar 6, PRRSV was identified in semen on approximately the same days as in serum; however, the semen viral loads were consistently higher than the serum viral load. This may indicate that the reproductive tract is a site of viral replication, and the virus may not go directly from the blood into the seminal compartment. Since there are differences in shedding durations and viral loads within the reproductive tracts of various boars, future studies correlating these levels with local immunity might be useful in determining the factors involved in the reduction and the elimination of viral loads.

ACKNOWLEDGMENTS

This project was funded by the National Pork Board/Iowa Pork Producers Council (grant 02-220); PIC USA; Tetracore, Inc.; Boehringer Ingelheim Vetmedica; Sygen International; and Animal Disease Research and Diagnostic Laboratory (ADRDL), South Dakota State University, Brookings.

Special thanks go to the Molecular Diagnostics Section of ADRDL, which includes Gina Steinlicht, Jessica Burton, Troy Lackey, Rebecca Hewer, and Shelly Weeks.

REFERENCES

1. **Altman, D. G.** 1991. Practical statistics for medical research. Chapman & Hall/CRC Press, Inc., Boca Raton, Fla.
2. **Benfield, D. A., E. A. Nelson, J. E. Collins, L. Harris, S. M. Goyal, D. Robison, W. T. Christianson, R. B. Morrison, D. Gorcyca, and D. Chladek.** 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Investig. **4:**127–133.
3. **Bierk, M. D., S. A. Dee, K. D. Rossow, J. E. Collins, M. I. Guedes, and T. W. Molitor.** 2000. Experiences with tonsil biopsy as an antemortem diagnostic test for detecting porcine reproductive and respiratory syndrome virus infection in breeding swine. Swine Health Prod. **8:**279–282.
4. **Cavanagh, D.** 1997. *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. Arch. Virol. **42:**629–633.
5. **Christopher-Hennings, J., E. A. Nelson, J. K. Nelson, R. J. Hines, S. L. Swenson, H. T. Hill, J. J. Zimmerman, J. B. Katz, M. J. Yaeger, C. C. L. Chase, and D. A. Benfield.** 1995. Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J. Clin. Microbiol. **33:**1730–1734.
6. **Christopher-Hennings, J., E. A. Nelson, R. J. Hines, J. K. Nelson, S. L. Swenson, J. J. Zimmerman, C. C. L. Chase, M. J. Yaeger, and D. A. Benfield.** 1995. Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. J. Vet. Diagn. Investig. **7:**456–464.
7. **Christopher-Hennings, J., E. A. Nelson, and D. A. Benfield.** 1996. Detecting porcine reproductive and respiratory syndrome virus in boar semen. Swine Health Prod. **4:**37–39.
8. **Christopher-Hennings, J., E. A. Nelson, J. K. Nelson, and D. A. Benfield.** 1997. Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. Am. J. Vet. Res. **58:**40–45.
9. **Christopher-Hennings, J., and E. A. Nelson.** 1997. PCR analysis for the identification of porcine reproductive and respiratory syndrome virus in boar semen, p. 81–88. *In* S. J. Meltzer (ed.), PCR in bioanalysis (Methods in Molecular Biology series). Humana Press, Totowa, N.J.
10. **Christopher-Hennings, J., E. A. Nelson, J. K. Nelson, K. R. Rossow, R. R. Rowland, M. J. Yaeger, C. C. L. Chase, R. Garduno, J. E. Collins, and D. A. Benfield.** 1998. Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and non-vasectomized boars. Vet. Pathol. **35:**260–267.
11. **Christopher-Hennings, J., L. D. Holler, D. A. Benfield, and E. A. Nelson.** 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. Investig. **13:**133–142.
12. **Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. Christopher-Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison, H. S. Joo, D. Gorcyca, and D. Chladek.** 1992. Isolation of swine infertility and respiratory syndrome virus (isolate VR-2332) in North America and experimental reproduction of the diseases in gnotobiotic pigs. J. Vet. Diagn. Investig. **4:**117–126.
13. **Conzelmann, K. K., N. Visser, P. Van Woensel, and H. J. Thiel.** 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group. Virology **193:**329–339.
14. **Coombs, R. W., C. E. Speck, J. P. Hughes, W. Lee, R. Sampoleo, S. O. Ross, J. Dragavon, G. Peterson, T. M. Hooton, A. C. Collier, L. Corey, L. Koutsky, and J. Krieger.** 1998. Association between culturable human immunodeficiency virus type 1 (HIV-1) in semen and HIV-1 RNA levels in semen and blood: evidence for compartmentalization of HIV-1 between semen and blood. J. Infect. Dis. **177:**320–330.
15. **Dunne, A. L., F. Mitchell, K. M. Allen, H. W. Gordon Baker, S. Garland, G. N. Clarke, A. Mijch, and S. M. Crowe.** 2003. Analysis of HIV-1 viral load in seminal plasma samples. J. Clin. Virol. **26:**239–245.
16. **Dyer, J. R., B. L. Gilliam, J. J. Eron, L. Grosso, M. S. Cohen, and S. A. Fiscus.** 1996. Quantitation of human immunodeficiency virus type 1 RNA in cell-free seminal plasma—comparison of NASBA™ reverse transcription-PCR amplification and correlation with quantitative culture. J. Virol. Methods **60:**161–170.
17. **Egli, C., B. Thür, L. Liu, and M. A. Hofmann.** 2001. Quantitative TaqMan RT-PCR for the detection and differentiation of European and North American strains of porcine reproductive and respiratory syndrome virus. J. Virol. Methods **98:**63–75.
18. **Gradil, C., C. Dubuc, and M. D. Eaglesome.** 1996. Porcine reproductive and respiratory syndrome virus: seminal transmission. Vet. Rec. **138:**521–522.
19. **Gupta, P., C. Leroux, B. K. Patterson, L. Kingsley, C. Rinaldo, M. Ding, Y. Chen, K. Kulka, W. Buchanan, B. McKeon, and R. Montelaro.** 2000. Human immunodeficiency virus type 1 shedding pattern in semen correlates with the compartmentalization of viral quasi species between blood and semen. J. Infect. Dis. **182:**79–87.
20. **Halbur, P. G., M. F. Rothschild, B. J. Thacker, X.-J. Meng, P. S. Paul, and J. D. Bruna.** 1998. Differences in susceptibility of Duroc, Hampshire and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). J. Anim. Breed Gene **115:**181–189.
21. **Horter, D. C., R. M. Pogranichniy, C.-C. Chang, R. B. Evans, K.-J. Yoon, and J. J. Zimmerman.** 2002. Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. Vet. Microbiol. **2315:**1–16.
22. **Hüssy, D., F. Janett, S. Albini, N. Stäuber, R. Thun, and M. Ackermann.** 2002. Analysis of the pathogenetic basis for shedding and transmission of ovine gamma herpesvirus 2. J. Clin. Microbiol. **40:**4700–4704.
23. **Kim, H. S., J. Kwang, K.-J. Yoon, H. S. Joo, and M. L. Frey.** 1994. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. **133:**477–483.

Downloaded from jcm.asm.org by on March 4, 2008

24. **King, G. J., and J. W. MacPherson.** 1973. A comparison of two methods for boar semen collection J. Anim. Sci. **36:**563–565.
25. **Krieger, J. N., R. W. Coombs, A. C. Collier, D. D. Ho, S. O. Ross, J. E. Zeh, and L. Corey.** 1995. Intermittent shedding of human immunodeficiency virus in semen: implications for sexual transmission. J. Urol. **154:**1035–1040.
26. **Mellors, J., L. A. Kingsley, C. R. Rinaldo, J. Todd, B. Hoo, R. P. Kokka, and P. Gupta.** 1995. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann. Intern. Med. **122:**573–579.
27. **Nelson, E. A., J. Christopher-Hennings, T. Drew, G. Wensvoort, J. E. Collins, and D. A. Benfield.** 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. **31:**3184–3189.
28. **Nielsen, T. L., J. Nielsen, P. Have, P. Bækbo, R. Hoff-Jøorgensen, and A. Bøotner.** 1997. Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. Vet. Microbiol. **54:**101–112.
29. **Ohlinger, V. F., F. Weiland, B. Haas, N. Visser, R. Ahl, T. C. Mettenleiter, L. Weiland, H. J. Rziba, A. Saalmuller, and O. C. Straub.** 1991. Der "seuchenhafte Spatabort beim Schwein"—ein Beitrag zur Atiologie des "porcine reproductive and respiratory syndrome (PRRS)." Tierarztl. Umschau. **46:**703–708.
30. **Prieto, C., P. Suarez, I. Simarro, C. Garcia, S. Martin-Rillo, and J. M. Castro.** 1997. Insemination of susceptible and preimmunized gilts with boar semen containing PRRSV. Theriogenology **47:**647–654.
31. **Quayle, A. J., C. Xu, K. H. Mayer, and D. J. Anderson.** 1997. T lymphocytes and macrophages, but not motile spermatozoa are a significant source of human immunodeficiency virus in semen. J. Infect. Dis. **176:**960–968.
32. **Ropp, S. L., C. Wees, Y. Fang, E. A. Nelson, K. D. Rossow, M. Bien, B. Arndt, S. Preszler, P. Steen, J. Christopher-Hennings, J. E. Collins, D. A. Benfield, and K. S. Faaberg.** 2004. Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. J. Virol. **78:**3684–3703.
33. **Rowland, R. R. R., S. Lawson, K. Rossow, and D. A. Benfield.** 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet. Microbiol. **96:**219–235.
34. **Shepard, R. N., J. Schock, K. Robertson, D. C. Shugars, J. Dyer, P. Vernazza, C. Hall, M. S. Cohen, and S. A. Fiscus.** 2000. Quantitation of human immunodeficiency virus type RNA in different biological compartments. J. Clin. Microbiol. **38:**1414–1418.
35. **Shin, J., J. Torrison, C. S. Choi, S. M. Gonzalez, B. G. Crabo, and T. W. Molitor.** 1997. Monitoring of porcine reproductive and respiratory syndrome virus infection in boars. Vet. Microbiol. **55:**337–346.
36. **Shin, J., E. M. Bautista, Y.-B. Kang, and T. W. Molitor.** 1998. Quantitation of porcine reproductive and respiratory syndrome virus RNA in semen by single-tube reverse transcription-nested polymerase chain reaction. J. Virol. Methods **72:**67–79.
37. **Sipos, W., C. Duvigneau, P. Pietschmann, K. Höoller, R. Hartl, K. Wahl, R. Steinborn, M. Gemeiner, M. Willheim, and F. Schmoll.** 2003. Parameters of humoral and cellular immunity following vaccination of pigs with a European modified-live strain of porcine reproductive and respiratory syndrome virus. Viral Immunol. **16:**335–346.
38. **Smith, R. D., B. Brown, P. Ikonomi, and A. N. Schechter.** 2003. Exogenous reference RNA for normalization of real-time quantitative PCR. BioTechniques **34:**88–91.
39. **Stöcher, M., and J. Berg.** 2002. Normalized quantification of human cytomegalovirus DNA by competitive real-time PCR on the LightCycler instrument. J. Clin. Microbiol. **40:**4547–4553.
40. **Sur, J.-H., A. R. Doster, J. S. Christian, J. A. Galeota, R. W. Wills, J. J. Zimmerman, and F. A. Osorio.** 1997. Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis and induces germ cell death by apoptosis. J. Virol. **71:**9170–9179.
41. **Swenson, S. L., H. T. Hill, J. J. Zimmerman, L. E. Evans, R. W. Wills, K.-J. Yoon, K. J. Schwartz, G. C. Anderson, M. J. McGinley, and A. K. Brevik.** 1994. Artificial insemination of gilts with PRRSV-contaminated semen. Swine Health Prod. **2:**19–23.
42. **Swenson, S. L., H. T. Hill, J. J. Zimmerman, L. E. Evans, J. G. Landgraf, R. W. Wills, T. P. Sanderson, M. J. McGinley, A. K. Brevik, D. K. Ciszewski, and M. L. Frey.** 1994. Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. J. Am. Vet. Med. Assoc. **204:**1943–1948.
43. **Umlauft, F., D. T. Wong, P. J. Oefner, P. A. Underhill, R. C. Cheung, T. L. Wright, A. A. Kolykhalov, K. Gruenewald, and H. B. Greenberg.** 1996. Hepatitis C virus detection by single-round PCR specific for the terminal 3′ noncoding region. J. Clin. Microbiol. **34:**2552–2558.
44. **Van Vugt, J. J. F. A., T. Storgaard, M. B. Oleksiewicz, and A. Bøotner.** 2001. High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. J. Gen. Virol. **82:**2615–2620.
45. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117–122.
46. **Wensvoort, G., C. Terprstra, M. J. M. A. Pol, E. A. der Laak, M. Bloemrad, E. P. deKluyer, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J. M. Broekhuijsen, P. L. J. M. Moonen, T. Zetstra, E. A. de Boer, H. J. Tibben, M. F. de Jong, P. Van't Veld, G. J. R. Groenland, J. A. van Gennep, M. T. Voets, J. H. M. Verheijden, and J. Braamskamp.** 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. **13:**121–130.
47. **Wills, R. W., J. J. Zimmerman, K. J. Yoon, S. L. Swenson, L. J. Hoffman, M. J. McGinley, H. T. Hill, and K. B. Platt.** 1997. Porcine reproductive and respiratory syndrome virus: routes of excretion. Vet. Microbiol. **57:**69–81.
48. **Wu, W.-H., Y. Fang, R. Farwell, M. Steffen-Bien, R. R. R. Rowland, J. Christopher-Hennings, and E. A. Nelson.** 2001. A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF 2b. Virology **287:**183–191.
49. **Xu, C., J. A. Politch, L. Tucker, K. H. Mayer, G. R. Seage III, and D. J. Anderson.** 1997. Factors associated with increased levels of human immunodeficiency virus type 1 DNA in semen. J. Infect. Dis. **176:**941–947.
50. **Yaeger, M. J., T. Prieve, J. Collins, J. Christopher-Hennings, E. A. Nelson, and D. A. Benfield.** 1993. Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. Swine Health Prod. **1:**5–9.

Downloaded from jcm.asm.org by on March 4, 2008