

Localization of Porcine Reproductive and Respiratory Syndrome Virus Infection in Boars by *In Situ* Riboprobe Hybridization

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ABSTRACT

The capability of porcine reproductive and respiratory syndrome virus (PRRSV) to be shed in semen for extended periods of time has been suggested to be a principal factor for viral transmission via insemination. In attempts to gain insights into the mechanism of PRRSV persistence in boars, tissue distribution and sites of viral infection were investigated by *in situ* hybridization (ISH) using digoxigenin-labeled RNA probe and the ISH results were compared with those of reverse transcription-nested polymerase chain reaction (RT-nested PCR). Animals were intranasally inoculated with 104 median tissue culture infectious dose of PRRSV VR-2332 and tissues collected at different times were examined. At day 7 postinfection, limited number of hybridization positive signals was observed in cells within or between seminiferous tubules in the testis sections while relatively abundant hybridization positive signals were observed in the brain stem and tracheobronchial lymph node. At later days of infection, hybridization positive signals were observed in cells within seminiferous tubules with much reduced frequency. Lack of agreement with the RT-nested PCR assay results in testis tissues obtained at days 14, 28, and 59 postinfection suggested that PRRSV infection in the testis may be extremely restricted, and may not necessarily constitute a major viral source in semen during extended periods of seminal shedding.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is recognized as an important disease of pigs that causes severe economic losses to the swine industry throughout the

world including North America, Europe, and Southeast Asia. The etiologic agent of PRRS was first reported in The Netherlands in 1991, named as 'Lelystad virus' [36] and in the USA in 1992, named as 'swine infertility and respiratory syndrome (SIRS) virus' [10]. Soon the isolated viruses were officially designated as PRRS virus (PRRSV). PRRSV is a single-stranded positive-sense RNA virus containing one genome of 15 kb in length [20]. PRRSV is now classified as the family of *Arteriviridae* under the newly established order of *Nidovirales* [3].

Various clinical signs ranging from subclinical to severe cases have been reported in naturally infected or experimentally infected pigs. Typical signs in sows and gilts are reproductive failures including late term abortions, stillbirths, early farrowings, and increased number of pigs born weak or dead [10, 36, 38]. In young pigs, high rates of preweaning mortality and frequent involvement of secondary infections are major features of the disease [10, 36, 38]. In boars, transient lethargy, depression, inappetence, mild pyrexia, loss of libido, abnormal sperm production have been reported [6].

PRRSV is shed in boar semen for extended periods of time [6, 28, 29, 34]. PRRSV shedding in semen has been suggested to be a principal factor in long distance transmission of the infection due to wide practice of artificial insemination [22, 23, 34, 37]. It has been reported that PRRSV infection causes significant reduction in semen quality in boars, such as decreased sperm motility and increased incidence of proximal or distal cytoplasmic droplets compared to semen from uninfected boars [7, 23, 28].

Formaldehyde-fixed, paraffin-embedded tissue specimens are routinely used for histological examinations mainly because of the high quality of cell and tissue morphology. Methods to detect PRRSV protein and RNA in formaldehyde-fixed, paraffin-embedded tissue specimens include immunohistochemistry and *in situ* hybridization (ISH). The detection of specific viral nucleic acids in infected cells and tissues by hybridization is being increasingly used as preferred means of studying cell and tissue tropism of virus infections due to the high specificity and potentially high sensitivity of the method. It is well known that formalin fixation can cause problems in immunohistochemistry by abolishing certain epitopes due to the excessive crosslinking. Moreover, primary antibodies used in immunohistochemistry have a potential to produce nonspecific staining, especially, in the inflammatory

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tissues enriched with cells expressing Fc receptors. Studies with cDNA probe specific for PRRSV ORF-7 RNA sequences have suggested that in situ hybridization assay for the detection of PRRSV RNA was highly specific as well as well-suited for application on formalin-fixed, paraffin-embedded tissue sections[1, 4, 5, 9, 14, 17, 18, 24-26, 31, 32, 35]. ISH using non-radioactive complementary RNA probes (riboprobes) has become a powerful technique for the examination of virus RNA or cellular gene expression in tissues[9, 21]. In this study, sites of PRRSV infection in boar tissues were investigated by in situ hybridization using digoxigenin-labeled riboprobe.

Materials and Methods

Viruses and cells

PRRSV VR-2332 was propagated in the CL-2621. Cells were seeded in LabTek 8-well tissue culture chamber slides for in situ hybridization. The cell monolayers were infected at a multiplicity of infection (moi) of 0.1. At 24 h postinfection, cells were fixed with 4% paraformaldehyde for 7 min and dehydrated in a series of graded ethanol. Two cytopins on each poly-L-lysine-coated slide (Sigma) were prepared from virus- and sham-inoculated 75 cm² flasks, and used as positive and negative controls.

Animals

A total of 10 boars were obtained from a commercial farm herd seronegative for PRRSV. Animals were individually housed and cared for according to guidelines of the Institutional Animal Care and Use Committee (IACUC), University of Minnesota. Nine boars were intranasally infected with 10⁴ median tissue culture infectious dose (TCID₅₀) of PRRSV VR-2332 per head. One boar served as uninfected control. Serum and semen samples were collected at an interval of 2 to 7 days from days 0 to 85 postinfection. Animals were anesthetized with Tilazol, and euthanized with overdose of pentobarbital sodium at days 0 (n=1), 7 (n=3), 14 (n=1), 28 (n=1), 57 (n=1), 59 (n=1), and 85 (n=2) postinfection. In this study, tissues obtained at day 7, 14, 28, and 59 were used for in situ riboprobe hybridization and RT-nested PCR assays.

Collection of semen and tissue preparation

Semen samples were collected into plastic bags covered with two layers of gauze to remove gel-like ejaculates. One milliliter aliquot was made and stored at -70 °C. Presence of PRRSV RNA in semen was tested by RT-nested PCR as previously described[28]. For in situ analyses, tissues, approximately 1 cm³ in size, were fixed in PLP fixative (2% paraformaldehyde containing 75 mM lysine and 2 mg/ml NaIO₄) overnight and embedded in paraffin. For RT-nested PCR assay, another portion of tissues, approximately 0.5 cm³ in size, were placed into TRIzol (Gibco BRL) and immediately frozen in a dry ice/ethanol bath. Tissue sections with 4 to 6 µm thick were cut on a microtome, floated on

diethylpyrocarbonate (DEPC)-treated water, and mounted on silane-coated slides (Sigma). Two to three serial sections were mounted on a slide for simultaneous sense and antisense riboprobe hybridization assays.

Preparation of digoxigenin-labeled sense and antisense riboprobes

Sense and antisense riboprobes specific for PRRSV VR-2332 RNAs were synthesized from the open reading frame (ORF)-7 gene sequence of VR-2332. A 328-bp fragment tagged with T7 promoter sequence at the 5' or 3' end of VR-2332 cDNAs was obtained using two-round PCR according to Birk and Grimm (1994)[2]. For generation of sense riboprobe, PRRSV VR-2332 cDNA was subjected to first-round PCR with primers VR7.1 (forward, 5'-ATG GCC AGC CAG TCA ATC A-3') and VR7.3 (reverse, 5'-TGA CGC GGA TCA GGC GCA C-3'). PCR products were column-purified. The second-round PCR was performed with primers H3-T7-VR7.1 (forward, 5'-CCA AGC TTC - taa tac gac tca cta tag gga ga - ATG GCC AGC CAG TCA ATC A-3') and VR7.2.2 (reverse, 5'-CGG ATC AGG CGC ACA GTA TG-3'). In vitro transcription was performed using 1 µg of purified PCR-derived templates. For generation of antisense riboprobe, antisense riboprobe template DNA was generated by the second-round PCR using primers VR7.2.1 (forward, 5'-CCA GTC AAT CAG CTG TGC CA-3') and H3-T7-VR7.2.2 (reverse, CCA AGC TTC - taa tac gac tca cta tag gga ga - CGG ATC AGG CGC ACA GTA TG-3'). Purified PCR products using a Chroma Spin-100 column (Clontech) were used as templates for in vitro transcription. Digoxigenin-11-UTP was incorporated into RNA strands synthesized from 1 µg PCR-derived templates using DIG RNA labeling kit (Boehringer Mannheim). All synthesized RNA probes were examined for labeling efficiency by comparing with labeled RNA from plasmid templates according to the manufacturer's instruction (Boehringer Mannheim). To compare the sensitivity of riboprobe with cDNA probe, a 296-bp long digoxigenin-labeled, single-stranded (ss) antisense DNA probe was generated by lambda exonuclease digestion of PCR products amplified with a pair of dephosphorylated and nondephosphorylated primers according to Hannon et al. (1993)[13].

In situ riboprobe hybridization

In situ riboprobe hybridization was performed according to Panoskaltsis-Mortari and Bucy (1995) [21] with slight modifications. Following deparaffinization and rehydration, tissue sections were heat-treated in a microwave oven for 12 min in 10 mM citrate buffer (pH, 6.0) according to Sibony et al. (1995) [30]. Post-heat fixation was performed with 4% paraformaldehyde-PBS for 20 min at room temperature. Following rinse in PBS twice, sections were digested with 20 µg/ml proteinase K in 20 mM Tris-HCl (pH, 8.0) and 2 mM CaCl₂ for 20 min at room temperature. To stop proteinase K digestion, sections were washed in 0.2%

glycine-PBS twice and in PBS once. Post-permeabilization fixation was performed in 4% paraformaldehyde-PBS for 5 min at room temperature. Sections were rinsed in PBS 3 times and in 2X standard sodium citrate (SSC) once. Following a brief air-dry to remove residual buffers on sections, 30 µl of hybridization solution containing 500 ng/ml of sense or antisense riboprobe (heat-denatured at 80 °C for 2 min), 50% deionized formamide, 4X SSC, 10% dextran sulfate, 500 µg/ml heat-denatured salmon sperm DNA, 200 µg/ml heat-denatured yeast tRNA, 2 mM EDTA, 1X Denhardt's solution was placed on each tissue section. Sections were covered with a silane-coated glass coverslip and tightly sealed with nail polish. Hybridization was performed in a humidified chamber overnight at 50 °C. The hybridization was followed by stringent posthybridization washings. The posthybridization washings included RNase A digestion of nonspecifically bound probes. The stringent washings and immunological detections were performed as described by Panoskaltsis-Mortari and Bucy (1995)[21].

Results

Specificity of *in situ* riboprobe hybridization

The specificity of sense and antisense riboprobe hybridization assays was tested using PRRSV-infected and fixed CL-2621 cell monolayers with or without RNase A treatment of target RNA before hybridization. While sense probe hybridization did not show any positive signals (Fig. 1, panel A & C), antisense probe hybridization produced intense positive signals on cells that were not disrupted of target RNA by RNase A (Fig. 1, panel B).

Effect of microwave heating or proteinase K pretreatment of cells and tissue sections

The microwave oven heating of tissue sections has widely been used as a method for antigen retrieval from formalin-fixed, paraffin-embedded tissues[27]. The enhancing effect of MW pretreatment on hybridization signal has been reported[30]. To optimize riboprobe hybridization conditions in this study, the effect of microwave oven heating and proteinase K pretreatment was examined using PRRSV VR-2332 infected CL-2621 cells and lung tissue sections obtained from a 7-day-infected, 6-week old pig intranasally exposed with VR-2332. In 4% paraformaldehyde-fixed CL-2621 monkey kidney cells, a sensitive hybridization signal was obtained by the combination of MW heating and proteinase K pretreatment (Fig. 2, panel A) or by proteinase K pretreatment alone (Fig. 2, panel B). However, proteinase K treatment alone (Fig. 2, panel B) produced higher background signal as compared to the combined treatment (Fig. 2, panel A). MW heat treatment alone of the CL-2621 cells by omitting proteinase K digestion did not produce sensitive

hybridization signal (data not shown). In PLP-fixed lung tissue sections, MW heating alone sufficiently produced specific hybridization signals with somewhat reduced intensity (Fig. 2, panel D) as compared to the combined treatment of lung sections (Fig. 2, panel C). From the results, MW heating followed by the proteinase K pretreatment of cells or tissue sections was effective for obtaining specific and sensitive hybridization signals with the need of acetylation step as suggested[30].

Localization of PRRSV RNA in boar tissues

Using the optimized conditions of riboprobe hybridization, the tissue distribution and cellular sites of PRRSV infection were examined in boars. In the nonreproductive tissues of the boar, hybridization positive signals were observed sparsely in the sections of the lung, TBLN, spleen, liver, heart, cerebrum, cerebellum, and brain stem tissues obtained at 7 dpi. In the lung, some positive signals were observed at alveolar septae and bronchiolar epithelial surface (data not shown). Relatively abundant positive signals were observed in the sections of brain stem and TBLN tissues (Fig. 3, panel A & B). The cross sections of brain stem tissues showed positive cells along with the capillary in the gray matter. TBLN sections showed positive cells in the paracortical area or in the germinal center.

In the reproductive tissues of the boar, tissues obtained at 7 dpi from the testis, epididymis, prostate gland, and bulbourethral gland showed positive signals. In the epididymis, some positive signals were observed in the inner epithelial lining (data not shown). In the testis, hybridization positive signals were observed in cells located in the interstitium adjacent to blood vessels between the seminiferous tubules (Fig. 3, panel C) or inside the seminiferous tubules (Fig. 3, panel D). Obvious nonspecific signals were observed in some population of cells in the seminiferous tubules when serial sections were examined with sense riboprobe or RNase A-pretreated sections (data not shown). The pictures shown in Fig. 3, panel C & D were taken from the regions where only antisense riboprobe hybridization produced positive signals.

To determine the cellular sites of PRRSV infection at later time postinfection after 7 dpi, two of the reproductive tissues (testis and epididymis) and three of the nonreproductive tissues (lung, TBLN, and brain stem) obtained at 14, 28, and 59 dpi were examined. Hybridization positive signals were observed in testis sections obtained at 14, 28, and 59 dpi, and epididymis sections at 14 and 59 dpi (Table 1). Hybridization positive signals were not observed in the lung and TBLN from the same boars from day 14 to 59 postinfection (Table 1). The brain stem showed a few positive signals on sections obtained at 59 dpi, but not at 14 and 28 dpi (Table 1). Sense and antisense riboprobe hybridizations on serial sections of testis tissues obtained at 28 and 59 dpi are shown in Fig. 4.

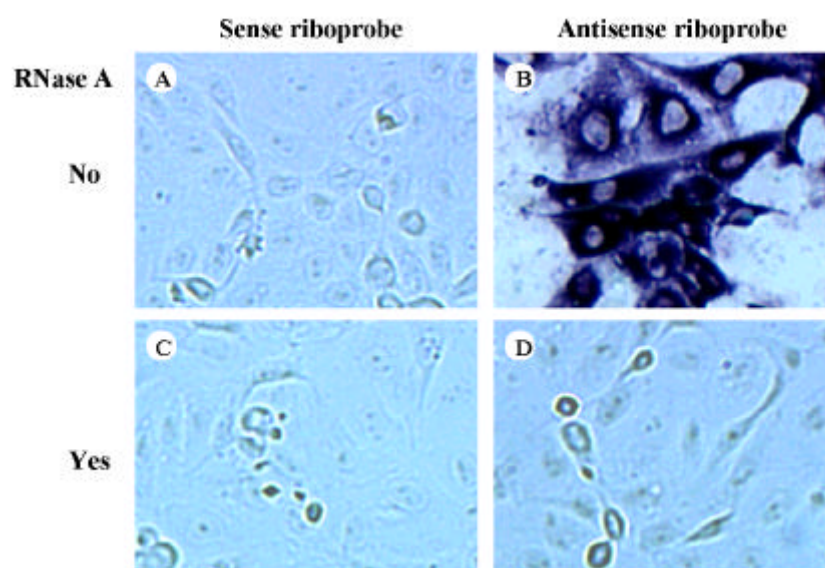


Fig. 1. Sense and antisense riboprobe hybridization with or without RNase A pretreatment of PRRSV-infected, fixed CL-2621 cells. CL-2621 monkey kidney cells infected with VR-2332 at a multiplicity of infection of 0.1 for 24 h were fixed in 4% paraformaldehyde for 7 min. After microwave heating and proteinase K treatment, hybridization was performed with PRRSV VR-2332 specific sense (A, C) and antisense (B, D) riboprobes on cells pretreated without (A, B) or with (C, D) 100 µg/ml RNase A at 37 °C for 30 min.

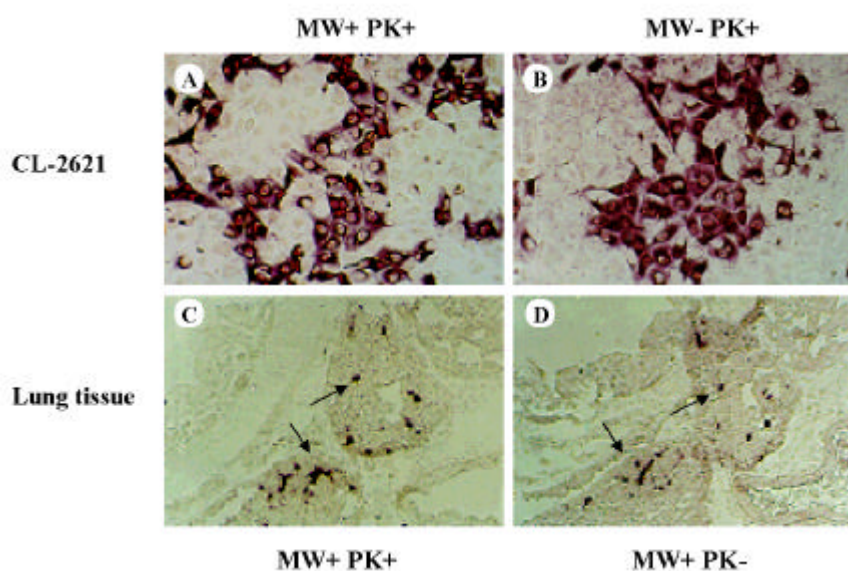


Fig. 2. Effect of microwave oven heating and proteinase K pretreatment of cells and tissues on in situ riboprobe hybridization signal. The effect of microwave (MW) oven heating and proteinase K (PK) pretreatment on riboprobe hybridization signal was examined using PRRSV VR-2332 infected CL-2621 monkey kidney cells (A & B) and lung tissue sections (C & D) of a 6-wk-old pig at day 7 postinfection. CL-2621 cells infected with VR-2332 and PLP-fixed, paraffin-embedded lung tissue sections were prepared as described in *Materials and Methods*. Acetylation of slides was performed for PK-pretreated slide (B). For MW heat-treated slides (A, C, and D), acetylation step was omitted according to Sibony et al. (1995) [30]. Hybridization was performed with antisense riboprobe specific for PRRSV VR-2332.

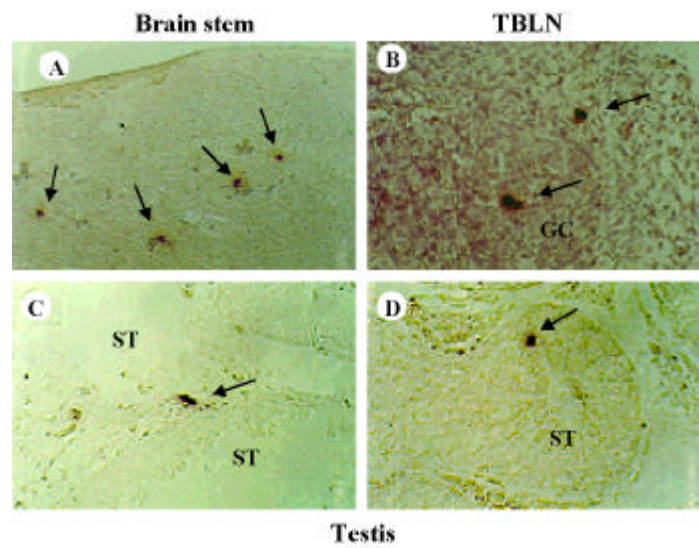


Fig. 3. Localization of PRRSV RNA in boar tissues obtained at day 7 postinfection. Riboprobe hybridization was performed on sections of tissues taken from PRRSV-infected boars at 7 dpi. Tissue sections were hybridized with digoxigenin-labeled, sense and antisense riboprobes (302-nt long) specific for PRRSV VR-2332 as described in *Materials and Methods*. All photographs were taken at 200X magnification from tissue sections of one representative boar. Riboprobe hybridization positive cells appear as dark violet/purple colors (arrows). Tracheobronchial lymph node (TBLN) section was counterstained with hematoxylin to elucidate the germinal center (GC). ST: seminiferous tubule.

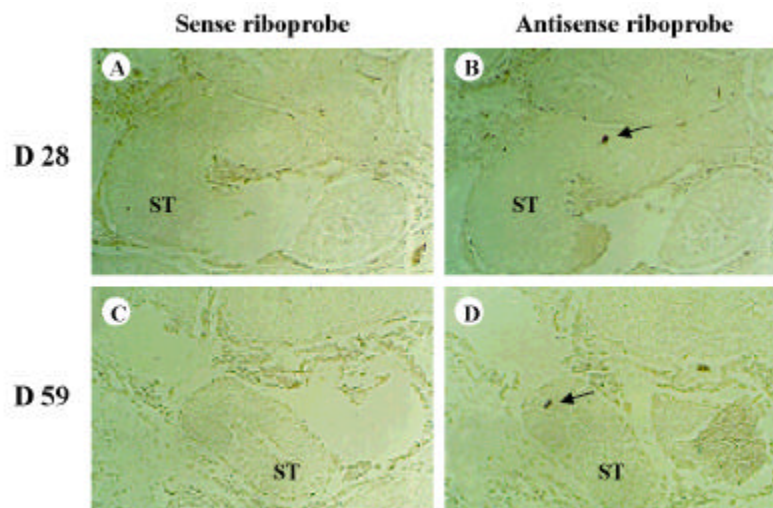


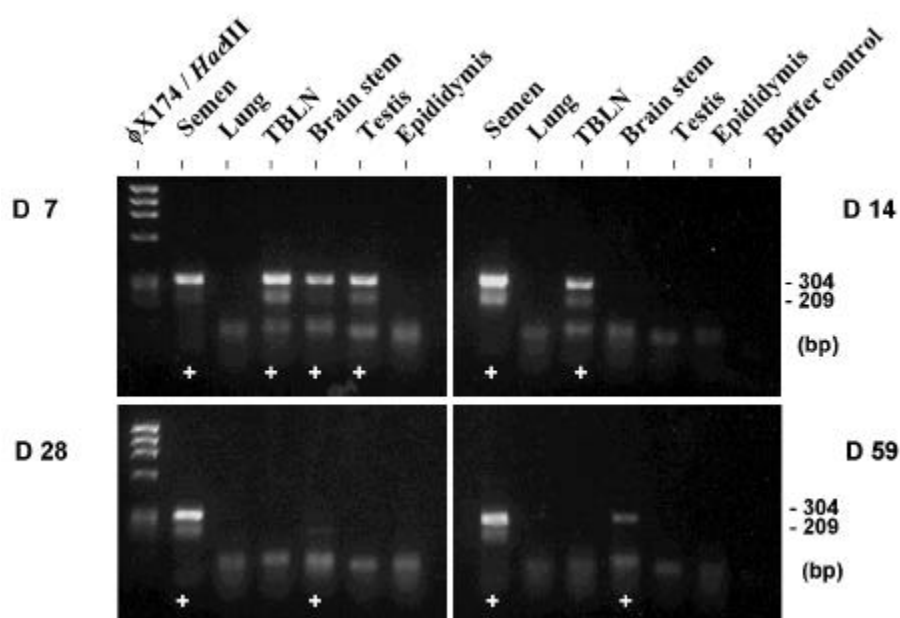
Fig. 4. Localization of PRRSV RNA on serial sections of testis tissues obtained at days 28 and 59 postinfection. Riboprobe hybridization was performed on sections of tissues taken from PRRSV-infected boars at 28 (A & B) and 59 dpi (C & D). Tissue sections were hybridized with digoxigenin-labeled, sense and antisense riboprobes (302-nt long) specific for PRRSV VR-2332 as described in *Materials and Methods*. All photographs were taken at 100X magnification. Antisense riboprobe hybridization positive cells appear as dark violet/purple colors (arrows). The photographs were taken from one of the representative results. ST: seminiferous tubule.

Table 1. A summary result of in situ riboprobe hybridization and RT-nested PCR assay in boar tissues obtained at the indicated days postinfection (dpi)

DPI	Boar	Seminal Shedding*	Nonreproductive tissue			Reproductive tissue	
			Lung	TBLN	Brain stem	Epididymis	Testis
7	A2	+	+ / - [†]	+ / +	+ / +	+ / -	+ / +
	B4	NA	+ / NT	+ / NT	+ / NT	- / NT	+ / NT
14	B6	+	- / -	- / +	- / -	+ / -	+ / -
28	A5	+	- / -	- / -	- / +	- / -	+ / -
59	A1	+	- / -	- / -	+ / +	+ / -	+ / -

*Seminal shedding of PRRSV was tested by RT-nested PCR.

[†] Results of PRRSV RNA detection by riboprobe hybridization and RT-nested PCR are indicated as positive (+) or negative (-). TBLN: tracheobronchial lymph node; NT: not tested; NA: not available.

**Fig. 5.** Detection of PRRSV in semen and selected tissues of infected boars at the indicated days postinfection by RT-nested PCR. Total cellular RNA was isolated from semen and TRIzol-preserved tissues. Each 1 µg total cellular RNA was subjected to RT-nested PCR assay. The amplified PCR products specific for PRRSV are seen as two distinct bands in size corresponding to the expected size from outer (304-bp) and inner (209-bp) primer pairs as described previously[28].

Detection of PRRSV by RT-nested PCR

RT-nested PCR assay was also performed using semen samples and selected tissues from 4 infected boars for comparison with the riboprobe hybridization results. Semen samples obtained at 7, 14, 28, and 59 dpi were positive for PRRSV RNA by RT-nested PCR test (Fig. 5). The overall results of hybridization and PRRSV PCR assay are summarized in Table 1. The TBLN (7 dpi), brain stem (7 and 59 dpi), and testis (7 dpi) showed positive results by

both assays. The lung (14, 28 and 59 dpi), TBLN (28 dpi), brain stem (14 dpi), and epididymis (28 dpi) showed negative results by both assays. A discrepancy between the results of the two assays was also observed. Despite the negative results of PCR assay in tissues taken from the epididymis (7, 14, 28, and 59 dpi) and testis (14, 28, and 59 dpi), positive results by PRRSV PCR assay were obtained in semen (7, 14, 28, and 59 dpi) and the brain stem (7, 28, and 59 dpi).

Discussion

In this study we investigated the tissue distribution and cellular sites of viral infection in boars infected with PRRSV using optimized conditions of sense and antisense riboprobe hybridizations. The results of hybridization were also compared with those of RT-nested PCR to obtain convincing evidence on tissue distribution of PRRSV infection. Previously, Sur et al. (1997) suggested that PRRSV replicates in testicular germ cells *in vivo*, results in the release of PRRSV-infected germ cells in semen, and alters spermatogenesis by the induction of testicular germ cell apoptosis. However, the present study on the localization of the cellular sites of viral infection in boars infected with PRRSV VR-2332 showed some inconsistent results as compared to the previously emphasized aspects on PRRSV infection in testicular germ cells and the induction of apoptotic germ cell death[33].

In boar tissues obtained at 7 dpi, hybridization positive cells were evident in the TBLN, brain stem, and testis as examined by digoxigenin-labeled antisense RNA probe specific for PRRSV VR-2332, and these results were in good agreement with PCR data. In testis sections, a few, yet distinct hybridization positive cells were localized in the areas of both testicular interstitium and inside seminiferous tubules, which are in good agreement with the previous findings[33]. However, the extremely rare number of hybridization positive cells and poor agreement with PCR data obtained at 14 to 59 dpi suggested that PRRSV progeny, if any, resulting from the infection of testicular cells may not necessarily constitute a major source of the virus in semen ejaculate, especially during persistent infection. Established or primary swine testicular cells did not support PRRSV replication *in vitro*. It will be intriguing to test whether PRRSV susceptibility resides in the differentiated state of testicular germ cells, or PRRSV gene expression depends on the local concentrations of sex steroids after puberty as indicated by sex hormone-dependent hepatitis B virus gene expression[11].

A study on the identification of PRRSV in semen and tissue of vasectomized and non-vasectomized boars with the same virus strain, ATCC VR-2332, suggested that the mechanisms of seminal shedding in intact and vasectomized boars were similar, in that both group of boars shed the virus in semen and that viral replication was most common within lymphoid tissue and macrophages were the predominant cell type containing PRRSV in both group of boars[8].

To date it is largely unknown why some immunocompetent adult boars become persistent carriers after infection with virulent strains of PRRSV. One possible explanation is that persistent PRRSV infection in the reproductive tracts of boars may be testosterone-dependent. Testosterone-dependent EAV persistence in the reproductive tracts of stallions has been proposed in that EAV persistence was not observed in castrated stallions or prepubertal colts[15]. Furthermore, testosterone supplementation of castrated stallions did not

show a significant drop in EAV load in semen or elimination of infectious virus from the reproductive tracts[19].

Another potential mechanism for PRRSV persistence in boars may result from PRRSV infection of immune-privileged sites during acute infection. It may be feasible that internal viral spread to the immune-privileged sites is extremely restricted due to several anti-inflammatory mechanisms exerted by immune-privileged sites against infection[12]. Paradoxically, the anti-inflammatory mechanisms of immune privilege may provide a favorable environment for the silent survival of PRRSV in the site, presumably by allowing the virus to evade specific immune recognition and to maintain its genome in low frequency of susceptible cells. In such case, there may exist technical difficulties to demonstrate infections in the sites.

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