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In Vivo Detection of Porcine Reproductive and Respiratory Syndrome Virus RNA by In Situ Hybridization at Different Times Postinfection†

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We studied the distribution of porcine reproductive and respiratory syndrome virus (PRRSV) RNA in tissues by in situ hybridization at different times postinfection (p.i.). The probe used for in situ hybridization was prepared by reverse transcription of PRRSV RNA, followed by PCR amplification of the cDNA. The sequence amplified corresponded to 433 bp from PRRSV open reading frame 7, which is contained in the nucleocapsid protein gene and which is highly conserved in both European and American strains (H. Mardassi, L. Wilson, S. Mounir, and S. Dea, J. Clin. Microbiol. 32:2197–2203, 1994). An immunohistochemical technique was used to detect PRRSV antigen in tissue from virus-infected animals by using a monoclonal antibody specific for the PRRSV nucleocapsid protein (E. A. Nelson, J. Christopher-Hennings, T. Drew, G. Wensvoort, J. E. Collins, and D. A. Benfield, J. Clin. Microbiol. 31:3184–3189, 1993). The detection of PRRSV RNA was conducted in tissues of 6-week-old pigs that had been infected with one of three different field PRRSV isolates and collected at times ranging from 4 to 42 days p.i. Hybridization signals specific for PRRSV RNA were detected in lung, lymphoid tissues, alveolar macrophages (obtained by lavage at the time of necropsy), Peyer's patches, and kidney. The PRRSV-positive cells in these tissues appeared to be predominantly macrophages. In lung tissue we also obtained evidence suggesting the involvement of type II pneumocytes in the replication of PRRSV. During the acute period of infection there was a close correlation between the detection of RNA and the detection of nucleocapsid protein in individual cells. At later times p.i. (28 and 42 days p.i.), instead, more cells containing only PRRSV RNA than those containing PRRSV RNA and also expressing PRRSV nucleocapsid protein were detected. These results suggest that PRRSV RNA might persist in the tissues of infected animals for a longer time than PRRSV antigen expression.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a spherical, enveloped virus classified in the genus *Arterivirus*, together with equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus (10, 28). The virion of PRRSV is 50 to 65 nm in diameter, with a central isometric nucleocapsid of approximately 30 to 35 nm in diameter. The genome of PRRSV is a single-stranded polyadenylated RNA molecule of positive polarity and of approximately 15 kb in length which contains eight open reading frames (ORFs). The nucleocapsid protein is encoded by ORF 7 and is highly conserved among both European and North American isolates (23, 25).

Epidemiological data indicate that reproductive and respiratory forms of porcine reproductive and respiratory syndrome are now endemic in domestic pigs of the United States and Europe. The acute pathogenesis of porcine reproductive and respiratory syndrome has been studied by numerous investigators by the use of virus isolation and immunohistochemical (IHC) techniques (9, 14–16, 18, 21, 29). However, little is known about the chronic effects of PRRSV infection. PRRSV is known to persist as a subclinical infection in individual animals for a long period of time (32) and therefore may be transmitted by contact or through semen to uninfected animals (2, 7, 8). Since the first application of in situ hybridization (ISH) to experimental virology in the early 1980s (13), this technique has been significantly improved and is now widely used because of its high degree of sensitivity and its ability to resolve viral gene expression. The development of ISH for the detection of PRRSV genomic RNA within infected tissue sections should make it possible to trace the path of the virus in the natural host throughout long postinfection (p.i.) periods with greater sensitivity.

A PRRSV-specific cDNA probe was produced by reverse transcription (RT)-PCR amplification of a segment of ORF 7 and was used to detect PRRSV nucleic acids in tissues and porcine alveolar macrophages (PAMs) of pigs infected intranasally with PRRSV. After infection of the pigs, PRRSV-positive cells were primarily distributed in the lung, lymphoid tissues, Peyer's patches of the ileum, and kidney. In particular, significant numbers of PRRSV-positive cells, morphologically consistent with macrophages, were detected in lung and lymphoid tissues throughout the study and up to the longest period studied (42 days p.i.).

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MATERIALS AND METHODS

Virus strains and cells. For optimization of ISH in cell cultures, we used the NVSL strain of PRRSV (obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Ames, Iowa) to inoculate MARC-145 cells (kindly provided by J. Kwang, Meat Animal Research Center, U.S. Department of Agriculture, Clay

Center, Nebr.). PRRSV strains 94-2367 and 1534 (kindly provided by David Benfield, South Dakota State University) and NEB-1 (provided by one of us [R. A. Hesse]) were used to infect pigs in several experiments of PRRSV strain pathogenesis which were the source of tissues for our study (11, 11a). MARC-145 cells (17) were infected at various multiplicities of infection. Viral identification was confirmed by indirect immunofluorescence in infected MARC-145 and in PAMs with monoclonal antibody (MAb) SDOW-17 (obtained from the National Veterinary Services Laboratory), which recognizes a conserved epitope of the PRRSV nucleocapsid protein (27).

Preparation of PRRSV-infected cell cultures for ISH. PRRSV-infected and noninfected MARC-145 cell cultures were used as the starting material for optimization of ISH. The cells were grown to confluency in Eagle's minimum essential medium with 5% fetal bovine serum. MARC-145 monolayers (75-cm^2 flasks) were infected with PRRSV at a multiplicity of infection of 1. The medium was removed when the cultures showed a 50 to 60% cytopathic effect, and the cells were suspended by trypsinization. The harvested cells were applied to Superfrost/plus slides (Fisher Scientific, Pittsburgh, Pa.) by centrifugation at $200 \times g$ for 5 min in a cytocentrifuge at a density of 10^5 cells per spot and were fixed in PLP (4% paraformaldehyde, 100 mM L-lysine dihydrochloride, 10 mM sodium *m*-periodate in phosphate-buffered saline [PBS; pH 7.4]) fixative for 5 min. The slides were then washed twice in PBS (pH 7.4) for 5 min and dehydrated through an ethanol series (70, 80, and 100%; 5 min at each concentration). The dehydrated slides were stored at 4°C until use.

Source of tissue samples and PAMs. Tissues fixed in 10% buffered-neutral formalin were obtained from two groups of 20 6-week-old pigs which were each inoculated with one of two field strains of PRRSV (PRRSV 94-2367 or PRRSV 1534, respectively) for a comparative study of PRRSV pathogenesis (11a). One group of 10 pigs remained uninfected and was used as a control group in the study. The pigs were inoculated intranasally with 3 ml (in each nare) of 10^4 50% cell culture infective doses of PRRSV per ml. Control pigs were inoculated in a similar manner with a mock-infected MARC-145 cell culture supernatant. PAMs and tissues were collected from one animal in each one of the three groups at days 4, 7, 14, and 42 p.i. Tissues from one control animal and from three animals of each PRRSV-inoculated group were collected on days 4, 7, and 14 p.i. Finally, tissues from five animals in each of the three groups were collected on day 42 p.i. Of these samples, tissues from three pigs of each group were randomly selected for ISH and IHC. In addition, tissues collected at 28 days p.i. during a separate experiment involving PRRSV NEB-1-challenged pigs (11) were also used for simultaneous ISH and IHC.

Extraction of genomic RNA. PRRSV NVSL-infected MARC-145 cells (75-cm^2 flask) were harvested when 70 to 80% of the cells were affected by cytopathic changes. RNA was extracted from infected cells with the Trizol LS Reagent (Gibco BRL, Grand Island, N.Y.) according to the manufacturer's instructions. The RNA contents of the PRRSV-infected MARC-145 extracts were estimated by measuring the A_{260} with a Milton Roy Spectronic 601 spectrophotometer. One microgram of total RNA extracted from PRRSV-infected MARC-145 cells was used as the template for RT. Total RNA was extracted from mock-infected MARC-145 cell cultures in a similar manner.

Primers. The primers from the PRRSV ORF 7 sequence amplification were published by Mardassi et al. (23). The sense (1010 PLS) and antisense (1011 PLR) primers were 5′-ATGGCCAGCCAGCCAGTCAATCA-3′ (nucleotides 51 to 69) and 5′-TCGCCCTAATTGAATAGGTG-3 (nucleotides 464 to 483), respectively. This primer pair has been shown to amplify both North American and European strains of PRRSV (22, 23).

RT-PCR. Twenty-five picomoles of antisense primer was added to 1 μg of extracted RNA in 7 μl of diethyl pyrocarbonate (DEPC; Sigma, St. Louis, Mo.)-treated water, denatured at 75°C for 10 min, and then cooled on ice. RT was performed in a final volume of 20 μl containing 1× RT buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 0.5 mM (each) deoxynucleotide triphosphates (dNTPs), 1 U of RNase inhibitor per μl, and 200 U of RNase H^- reverse transcriptase (Gibco BRL). After incubation for 1 h at 42°C, the mixture was heated at 95°C for 5 min and chilled on ice. A PCR was set up with 20 μl of the RT product in 100 μl of 1× PCR buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM [each] dNTPs, and 25 pmol of each of the sense and antisense primers). After the addition of 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) and a thin layer of mineral oil (Sigma), the PCR mixtures were cycled 35 times through denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min as described by Mardassi et al. (23). For product visualization, 10 μl of the amplified RT-PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The 433-bp cDNA fragment was purified from the agarose gel with GENECLEAN (Bio 101, Inc., La Jolla, Calif.).

Labeling and specificity control of the cDNA probe. The cDNA probe specific for a region of ORF 7 in PRRSV was labeled by a random priming reaction with digoxigenin-dUTP (DIG; Boehringer Mannheim Corp., Indianapolis, Ind.). This cDNA represents a region of the PRRSV genome that is present in all subgenomic mRNAs and therefore provides a high degree of sensitivity by hybridizing with all species of PRRSV RNA (sense and antisense genomic RNAs and subgenomic mRNAs) present in infected cells.

The specificity of the labeled cDNA probe was confirmed by slot blot hybridization with RNA fractions obtained from PRRSV-infected and mock-infected MARC-145 cells by using a Bio-Rad 48-well slot format Bio-Dot SF apparatus and Zeta-Probe membrane (Bio-Rad, Herculeus, Calif.).

Pretreatment of slides for ISH. All solutions used for pretreatment, prehybridization, and hybridization were treated with 0.1% DEPC-treated water. Cells and tissue sections (5-μm thickness) were rehydrated and equilibrated in PBS for 3 min. Deproteinization was carried out in 0.2 N HCl for 20 min at room temperature; this was followed by washing with DEPC-treated water for 1 min and by digestion with proteinase K (Gibco BRL) at 4 μg/ml for cells and 20 μg/ml for tissues for 20 min in PBS at 37°C. Serial sections of each infected tissue sample were treated with RNase A (Boehringer Mannheim) at 100 μg/ml in 10 mM Tris-Cl (pH 7.4) for 30 min at 37°C to remove target RNA as a specificity control. After digestion, all cells and tissues were fixed in 4% paraformaldehyde in PBS for 5 min. After rinsing with PBS twice, the slides were acetylated in 300 ml of 0.1 mM triethanolamine-HCl buffer (pH 8.0) to which 0.75 ml of acetic anhydride (0.25%) was added. After 5 min, 0.75 ml of acetic anhydride was added for an additional 5 min and the slides were then rinsed in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]).

ISH. The prehybridization mixture contained 50% deionized formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt's solution (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 2 mM EDTA, and 500 μg of salmon testis DNA (Sigma) per ml. The slides were covered with 200 μl of prehybridization mixture and were incubated in a humidified chamber for 1 h at 65°C. A heterologous cDNA probe of 346 bp from the pseudorabies virus gG glycoprotein gene was also labeled with DIG and was applied to the PRRSV-infected tissues as a specificity control. The labeled probes (0.1 ng/μl) were diluted in 300 μl of the prehybridization mixture and then heated for 5 min in a 95°C heating block and quenched on ice before being applied to the cells and tissue sections. The slides were rinsed briefly in 2× SSC, and 25 μl of probe mixture for cells and 70 μl of probe mixture for tissues was applied to each slide. The hybridization was performed overnight at 56°C. After hybridization, the cells and tissue sections were washed twice in 4× SSC for 5 min at room temperature, once in 2× SSC for 10 min at 37°C, once in 0.2× SSC containing 60% formamide for 10 min at 37°C, twice in 2× SSC for 5 min at room temperature, twice in 0.2× SSC for 5 min at room temperature, and once in buffer I (100 mM maleic acid, 150 mM NaCl [pH 7.5]) for 5 min at room temperature.

Immunohistological detection of hybridized cDNA probe. To reduce background staining the slides were preincubated with buffer II (1% blocking reagent in buffer I; Boehringer Mannheim) for 40 min at room temperature. The anti-DIG–alkaline phosphatase conjugate (Boehringer Mannheim) was freshly diluted to 1:500 in buffer II and was then added to the cells and tissue sections. This incubation was carried out for 1 h in a humidified chamber at room temperature. All incubations were followed by washes in two changes of buffer I for 5 min at room temperature and once in buffer III (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ [pH 9.5]) for 5 min at room temperature. Finally, the cells and tissue sections were incubated with color substrate solution. The color solution consisted of 45 μl of nitroblue tetrazolium salt (75 mg/ml in 70% dimethyl formamide) and 35 μl of 5-bromo-4-chloro-3-indolylphosphate, toluidinium salt (X-phosphate; 50 mg/ml in dimethyl formamide), in 10 ml of buffer III. The reaction was incubated for 3 to 8 h in the dark. The color reaction was stopped with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Counterstaining was done with 0.5% methyl green, and the slides were then washed with distilled water for 1 min and dried completely. The slides were dipped in xylene, and then cover slips were applied with a mounting solution of polyvinyl alcohol-vinyl acetate (Permount) before microscopic examination.

IHC. Paraffin-embedded sections were cut to thicknesses of 4 to 5 μm, placed on Superfrost/plus slides, and stained by a modification of the procedure of Halbur et al. (14). Briefly, endogenous peroxidase activity was removed by one 30-min incubation in 3% hydrogen peroxide in PBS (pH 7.4) and then a wash in Tris buffer (50 mM Tris buffer [pH 7.6]); tissue sections were then digested with 0.05% protease (Protease XIV; Sigma) in Tris buffer for 5 min at 37°C. Nonspecific antibody binding was blocked by incubation with 1.5% normal goat serum (Sigma). For primary immunostaining of PRRSV nucleocapsid antigen (MAb SDOW-17), sections were incubated with the MAb (ascitic fluid, diluted 1:500) or normal goat serum (negative control) overnight at 4°C; this was followed by incubation for 45 min at room temperature with biotinylated goat anti-mouse linking antibody (Dako Corp., Carpinteria, Calif.) for 30 min. The sections were then treated with peroxidase-conjugated streptavidin (Dako Corp.) for 30 min. Finally, the sections were incubated with 0.016% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.05% H_2O_2 for 4 to 5 min at room temperature and were counterstained with Mayer's hematoxylin-eosin. The slides were covered with cover slips by using mounting medium (Polysciences, Inc., Warrington, Pa.). For primary immunostaining of swine macrophages, sections of spleens and other lymphoid tissues from healthy pigs were incubated with different dilutions (1:100 through 1:1,000) of ascitic fluid from two hybridomas producing MAbs against swine macrophages which are available from the American Type Culture Collection (3).

Virological assays. Assessment of viremia and infectivity in tissue homogenates was carried out on the samples taken at days 4, 7, 14 and 42 days p.i. from three animals in each of the two inoculated groups (PRRSV 94-2367 and PRRSV 1534). For these assays the samples were processed and inoculated onto monolayers of PAMs in 96-well, flat-bottom microtiter plates as described previously (11).

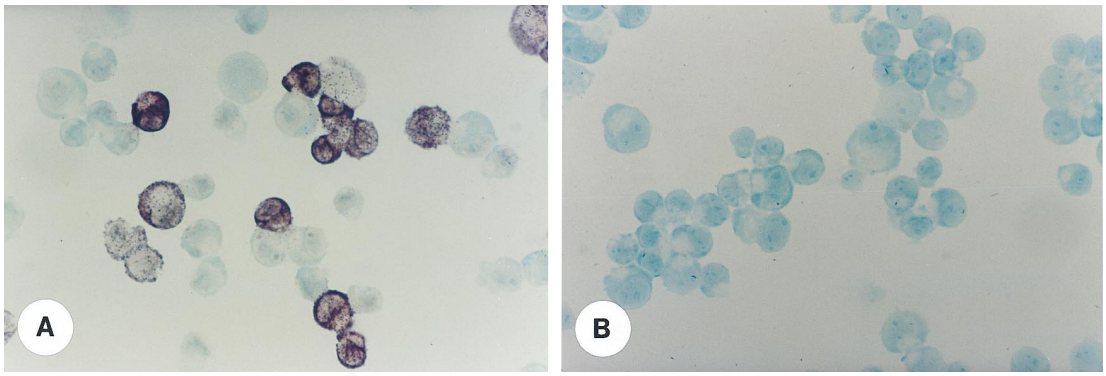

FIG. 1. (A) PRRSV-infected MARC-145 cells hybridized with a DIG-labeled cDNA probe (433 bp) generated by RT-PCR from PRRSV ORF 7. (B) ISH of mock-infected MARC-145 cells with the same probe used for panel A. Methyl green counterstain was used for both panels. Magnifications, ×625.

RESULTS

Experimental inoculations. Compared with the uninoculated controls, a remarkable clinical observation in the PRRSV 94-2367- and PRRSV 1534-inoculated groups consisted of increased rectal temperature within 12 h of inoculation, which remained elevated for approximately 20 days p.i. in one animal (in the PRRSV 94-2367-inoculated group) and 9 days p.i. in another animal (in the PRRSV 1534-inoculated group). In both animals other clinical signs were similar to those described previously in pigs of a similar age experimentally inoculated with the PRRSV NEB-1 strain (11) and consisted of lethargy, moderate respiratory dyspnea, and cutaneous erythema. Of the two PRRSV-inoculated groups, clinical signs were consistently more prominent in the PRRSV 94-2367-inoculated group (11a). Significant gross pathological lesions consisted of moderate to marked enlargement of bronchial lymph nodes. Lung pathology was observed within 4 days p.i. and increased in severity to 14 days p.i. A diffuse tan discoloration and consolidation were consistent findings in both cases.

ISH and IHC signals. PRRSV sequences were detected by use of an anti-DIG–alkaline phosphatase conjugate when PRRSV-infected MARC-145 cells were hybridized with a specific DIG-labeled PRRSV cDNA (433-bp) probe. The PRRSV nucleic acid signal was specific because this was completely absent from mock-infected MARC-145 cells (Fig. 1A and B). ISH was used to determine the time of appearance of PRRSV-positive signals in PAMs obtained by bronchial lavage performed at the time of necropsy of inoculated and control pigs. The PRRSV-infected PAMs were detected as early as day 4 p.i. The alkaline phosphatase precipitate was observed in the cytoplasm within these labeled PAMs (Fig. 2A). The positive ISH signal in PAMs was most frequently seen between 4 and 7 days p.i., when 4 to 10 positive cells per 10^5 cell spots were observed. The signal then decreased until 14 days p.i. (to 1 cell per 10^5 cell spots) and became undetectable afterward.

In tissues, the most abundant and consistent ISH signal was observed in lung, especially at 4 and 7 days p.i. (Fig. 2B and D). The positive cells typically displayed dark purple-staining deposits in the cytoplasm. The location and morphology of the most frequent PRRSV-positive cells in the lung seem to indicate that they were in alveolar macrophages. This observation was also confirmed with consecutive serial sections stained with hematoxylin-eosin (data not shown). In several cases, the signal also coincided with a cell morphology and location that would suggest that the cell could be classified as a type II pneumocyte (Fig. 2B). In all cases the specificity of the signal reaction was confirmed by the rigorous observance of three specificity controls: (i) predigestion of a consecutive tissue section with a solution of RNase A prior to the performance of ISH which precluded or significantly decreased the development of the ISH signal, (ii) ISH performed on consecutive sections of infected tissues with a pseudorabies virus heterologous probe which likewise showed no positive signal, and (iii) ISH performed with a PRRSV probe on tissues of uninfected pigs which were consistently negative (data not shown). In addition, proof of specificity was provided by the performance of IHC on sections immediately consecutive to those on which ISH was performed. The thickness of the section used (5 μm) ensured that most positive cells would be present in both consecutive sections. The performance of IHC and ISH on serial sections of lungs indicated a very close cell-to-cell correlation between the signal of both techniques when they were performed on samples taken during the acute p.i. period with any PRRSV strain (4 and 7 days p.i.) (Fig. 2C and D). However, when this simultaneous detection was performed at late p.i. periods (at 28 days p.i. with PRRSV NEB-1-inoculated animals [11] and at 42 days p.i. with PRRSV 1534- and 94-2367-inoculated animals), the signal by IHC was significantly decreased in a number of cells. The IHC-positive cells were also positive by ISH, while many additional positive cells were only evident in the ISH section (Fig. 2E and F).

In an attempt to confirm the identities of macrophages identified by histological morphology, we performed IHC on sections of tissues using two MAbs specific for swine macrophages. However, these MAbs were unsuccessful in staining macrophage-rich areas of spleen, lung, and other swine tissues (data not shown). In addition to lung tissue, we demonstrated by ISH that between 4 and 42 days p.i. many PRRSV-infected cells are localized in macrophage-rich areas of other tissues including tonsil, thymus, bronchial lymph node, spleen, Peyer's patches of the ileum, and kidney. In the lung tissue, the histopathological changes were characterized by multifocal proliferative interstitial pneumonia, septal infiltration with mononuclear cells, and accumulation of macrophages with time (data not shown). Bronchial lymph nodes from many of the PRRSV-challenged pigs contained positive cells within lymphoid follicles, while a few positive cells were scattered in the paracortical regions near germinal centers (Fig. 3A). PRRSV-positive cells in the thymus were observed both in the thymic medulla and the thymic cortex (Fig. 3B). PRRSV-positive cells observed in tonsillar sections taken during the acute phase (4, 7, and 14 days p.i.) were found in both the tonsillar follicle and the crypt epithelium (data not shown). Unlike other lymphoid tissues, the spleen had only a few positive cells in germinal centers of lymphoid nodules (data not shown).

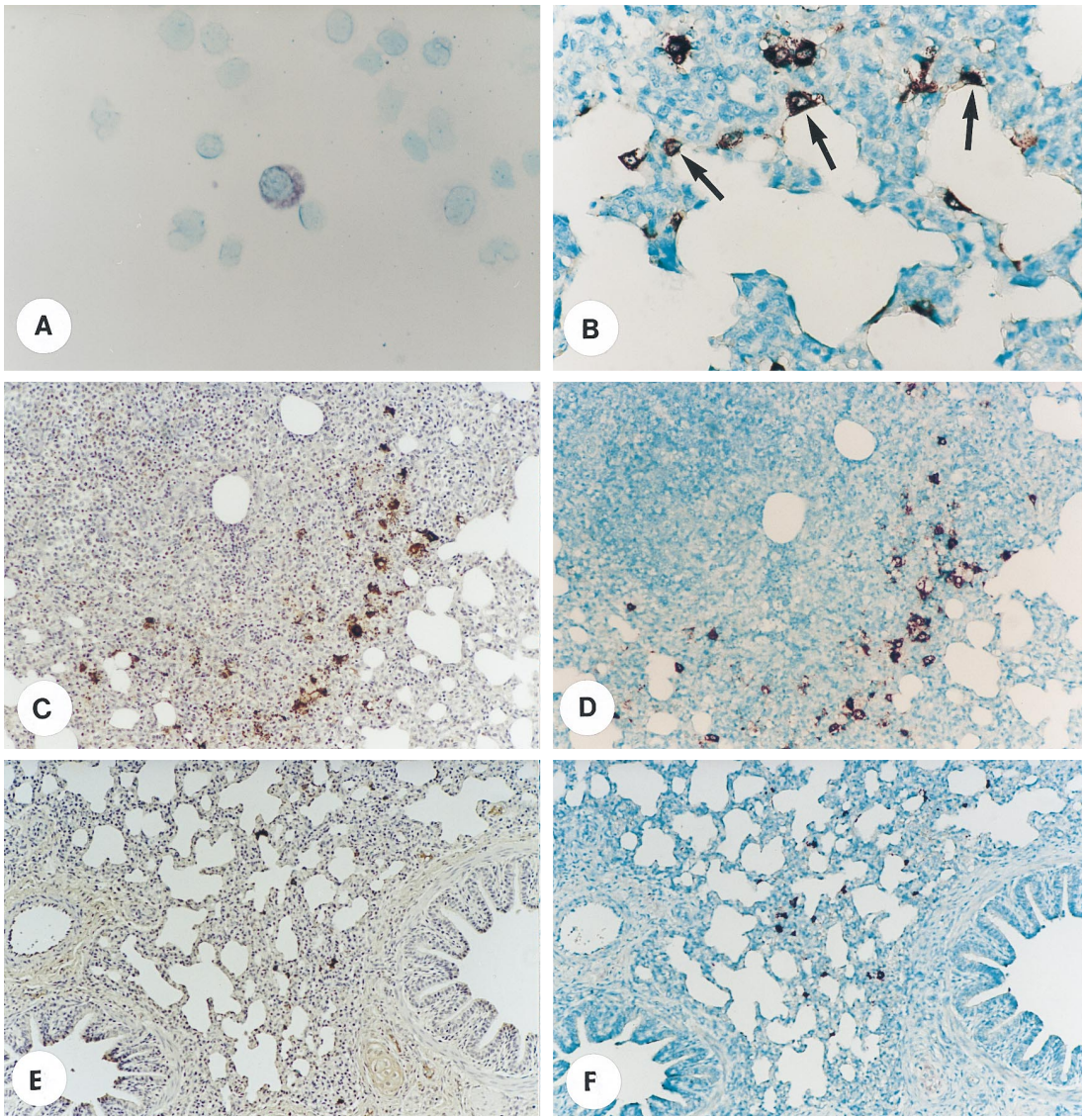

FIG. 2. (A) ISH performed on PAMs obtained by bronchial lavage upon necropsy of a PRRSV-infected pig at 7 days p.i. A positive ISH signal in the cytoplasm of one PAM is shown in the center. Methyl green counterstain was used. Magnification, ×625. (B) PRRSV-infected cells in the lung of a pig at 7 days p.i. with PRRSV. Large numbers of positive cells free in alveolar spaces and within the alveolar septae can be seen. Arrows indicate cells that by morphology and location could be classified as type II pneumocytes. ISH was performed, and methyl green counterstain was used. Magnification, ×625. (C and D) Serial sections of the PRRSV-infected lung tissue taken from an infected pig at 4 days p.i. (C) PRRSV-infected cells in alveolar septae. Immunoperoxidase with hematoxylin counterstain was used. Magnification, ×250. (D) Serial section (5-μm thickness) of the cells in panel C processed by ISH for PRRSV RNA showing that the same cells positive for PRRSV antigen (C) also contained PRRSV RNA. Methyl green counterstain was used. Magnification, ×250. (E and F) Serial sections of PRRSV-infected lung tissue taken from PRRSV-infected pig at 28 days p.i. (E) A few PRRSV-infected cells in alveolar septae. Immunoperoxidase with hematoxylin counterstain was used. Magnification, ×200. (F) Serial section of the cells in panel E but processed by ISH for PRRSV RNA, showing that more abundant PRRSV-infected cells are present, in addition to those positive for PRRSV antigen in panel E. Methyl green counterstain was used. Magnification, ×200.

PRRSV-positive cells observed in the kidney were found predominantly in the renal medulla. These cells may be interstitial macrophages (Fig. 3C). Additionally, PRRSV-positive cells were observed within Peyer's patches of the ileum (Fig. 3D). Positive cells appeared to decrease in number after 14 days p.i. At 42 days p.i. the only tissues in which viral RNA was most consistently detected were the lung and tonsil (Fig. 3E & F).

Viral infectivity in tissues. The results of isolation of infectious PRRSV from replicates of the tissue samples analyzed by ISH are presented in Table 1. In the case of both PRRSV-inoculated groups (PRRSV 94-2367 and 1534), the isolation of infectious PRRSV from tissue homogenates followed a pattern consistent with that previously observed during acute replication of NEB-1 (11) and other PRRSV field isolates (24). Positive viral isolation from lung samples collected at 28 days after infection with PRRSV NEB-1 has been reported previously (11). Attempts to isolate virus from samples from the uninoculated control group were negative (data not shown).

DISCUSSION

Here we report the detection of PRRSV RNA in tissue sections and PAMs from pigs infected intranasally with this virus and collected at different times, which ranged from 4 to 42 days p.i. ISH allowed us to detect PRRSV-specific RNA,

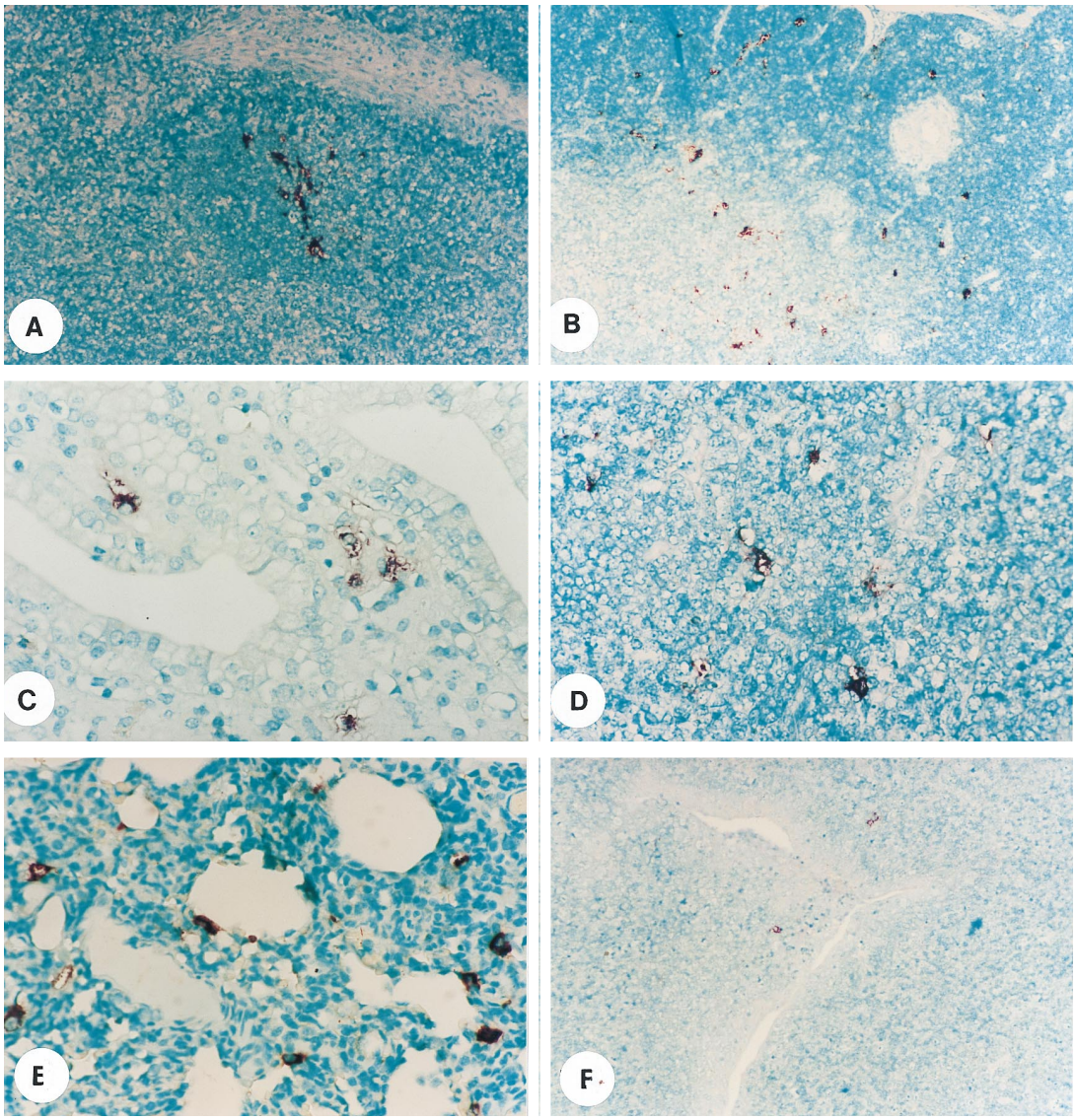

FIG. 3. (A) Positive ISH signals in germinal center of bronchial lymph node of a pig at 4 days p.i. with PRRSV. Methyl green counterstain was used. Magnification, ×250. (B) Positive ISH signals in thymus of a pig at 4 days p.i. with PRRSV. Positive cells are located both in the thymic medulla and the thymic cortex. ISH was performed, and methyl green counterstain was used. Magnification, ×250. (C) PRRSV-infected cells in the kidney of a pig at 7 days p.i. with PRRSV. Positive cells are located in the interstitium of the renal medulla. ISH was performed, and methyl green counterstain was used. Magnification, ×625. (D) PRRSV-infected cells in the ileum of a pig at 7 days p.i. with PRRSV. Positive cells are located in Peyer's patches. ISH was performed, and methyl green counterstain was used. Magnification, ×625. (E) PRRSV-infected cells in the lung of a pig at 42 days p.i. with PRRSV. Positive cells are located in the alveolar septae. ISH was performed, and methyl green counterstain was used. Magnification, ×625. (F) PRRSV-infected cells in the tonsil of a pig at 42 days p.i. with PRRSV. Positive cells are located in tonsillar follicle. ISH was performed, and methyl green counterstain was used. Magnification, ×250.

and its specificity was clearly established by appropriate controls. The tissue with the highest signal intensity for PRRSV infection at all times p.i. was lung. The localization of positive signals in lung tissue was most abundant in the cytoplasm of alveolar macrophages. This is consistent with what has been previously reported by other laboratories on the basis of the use of IHC (14, 15, 18, 20) and also our own IHC studies reported here.

The extent of ISH signals in macrophages of lung sections at early periods p.i. suggests that PAMs are a prime target of PRRSV infection. However, in our studies PAMs obtained by lavage did not exhibit, even at early periods of infection, a significant frequency of positive cells. This low ratio of ISH-positive PAMs does not necessarily reflect an in vivo situation; it is possible that the procedure that we used to cytospin and fix the PAMs for ISH selected against the attachment and retention of infected cells fixed on the slides. The possible involvement of other respiratory cells (i.e., type II pneumocytes) in the replication of PRRSV in the lung has been previously suggested by IHC (14, 20). In addition, extensive hypertrophy and hyperplasia of type II pneumocytes are known to occur during acute PRRSV infection of the lung (6, 12, 16, 30). Our ISH results give support to a role of type II pneumocytes in the replication of PRRSV, because the lung sections in our experiments showed cells which exhibited positive ISH signals and which morphologically and topographically are consistent with a type II pneumocyte identity (Fig. 2B). However, an unequivocal confirmation of pneumocytes as a site of replication of

TABLE 1. Detection of PRRSV 94-2367 and 1534 by ISH or infectivity assay

PRRSV strain	Tissue	Finding by the indicated assay on the following day p.i.[b]:							
		4		7		14		42	
		VI	ISH	VI	ISH	VI	ISH	VI	ISH
94-2367	Serum	+	NA	+	NA	+	NA	−	NA
	Thymus	+	+	+	−	+	ND	−	−
	Heart	−	−	−	−	−	ND	−	−
	Lung	+	+	+	+	+	+	−	+
	Spleen	−	+	−	±	−	±	−	−
	Bronchia lymph node	+	+	+	+	+	+	−	−
	Tonsil	+	+	+	+	+	+	−	+
1534	Serum	+	NA	+	NA	+	NA	−	NA
	Thymus	+	+	+	+	+	+	−	−
	Heart	+	−	+	−	−	ND	−	−
	Lung	+	+	+	+	+	+	−	+
	Spleen	+	+	+	+	+	+	−	−
	Bronchia lymph node	+	ND	+	ND	+	+	−	−
	Tonsil	+	ND	+	ND	−	+	−	+

[a] Viral isolation (VI) assays were conducted on tissues collected from two groups of pigs inoculated with either PRRSV 94-2367 or PRRSV 1534. Each observation was made on samples collected from three animals also analyzed by ISH.

[b] +, at least one of three animals was positive or negative by viral isolation or ISH signal; ±, weak ISH signal; ND, not done; NA, not applicable.

PRRSV will require further study of these positive cells by other methods such as ultrastructural analysis by electron microscopy of the same tissue section used for ISH (that work is in progress in our laboratory).

During the early phase of infection, PRRSV-positive cells detected by ISH were noticeably more abundant in the lung than in lymphoid tissues and kidney. The early appearance in the lung of PRRSV RNA parallels reports of others of the early isolation of infectious PRRSV from lung tissue (9, 29) and correlates well with our own viral isolation assays (Table 1). This close temporal relationship between infectious virus and viral RNA could suggest a possible direct viremia caused by the replication of PRRSV in pulmonary macrophages which would result in the release of PRRSV into the vascular compartment.

The controls included in our ISH protocol confirm the specificity of our PRRSV RNA in situ detection methodology. More importantly, other compelling evidence that confirms the specificity of our ISH protocol was provided by our simultaneous use of ISH and IHC with serial sections of the same tissue. The IHC technique that we used was standardized and described by previous investigators (14) and is based on the detection of the PRRSV nucleocapsid antigen. It is remarkable that during the acute phase of PRRSV infection many infected cells were detected by IHC in the alveolar septae of the lung and that these were in absolute correlation with the location of these same cells containing PRRSV RNA determined by ISH in the consecutive section. In this respect, we can say that the sensitivities of ISH and IHC are equivalent and acceptable for the detection of infected cells in the tissues of acutely infected animals. This is perhaps due to the abundance of both PRRSV antigens and genomic and subgenomic PRRSV RNAs in cells actively replicating virus in the lung during early periods p.i. However, when we compared both techniques at longer periods p.i., at which it is possible that a lower level of replication takes place, it became obvious that ISH is more sensitive and specific than IHC. We base this statement on the comparison of consecutive serial sections at 28 days p.i. (Fig. 2E and F) with one strain of PRRSV. The results obtained with sections at 42 days p.i. with another strain of PRRSV (94-2367) were consistent with this interpretation (data not shown). The difference in sensitivity between both techniques could presumably be due to intrinsic differences of the techniques in question. It is well known that antigen immunodetection requires significantly higher numbers of target molecules than nucleic acid hybridization to reach a specific signal which would be consistent with a positive reaction (31). This was particularly difficult to achieve by IHC because as the number of positive cells decreased, the differentiation between signal and noise became more difficult because of the higher level of background staining that is characteristic of IHC reactions. Alternatively, it may be argued that the superior sensitivity of ISH over IHC at longer times p.i. is based on the fact that antigen production in many of these infected cells has ceased or fallen below the levels of detection, while PRRSV RNAs persisted in the cells. Examples of the in vivo persistence of viral RNA upon acute infection linked to minimal or no expression of structural viral antigens are well known (1, 5, 19, 26). Further studies on the transcriptional activity of PRRSV during persistence by using single-stranded probes for ISH will certainly clarify this issue. Our results indicated that the number of cells supporting PRRSV infection during the late or persistent phase is limited, these being most noticeable in lung and tonsils. In fact, at these periods p.i. the ISH assay was able to detect PRRSV RNA while viral isolation attempts were negative (Table 1). This could also provide support to the concept of the persistence of PRRSV at late periods p.i. in a noninfectious form. However, it should be mentioned that routine assessment of infectivity in tissue homogenates like the method that we used in the experiments described here are less sensitive than methods involving cocultivation of the target cells (i.e., alveolar macrophages obtained by lavage) with indicator cells, as described by Mengeling et al. (24). It is possible, then, that if we had optimized the viral isolation procedures we would have detected infectious PRRSV at longer times p.i. PRRSV-positive signals in the kidney were observed within cells morphologically resembling and whose location was consistent with interstitial macrophages in the renal medulla at 4 and 7 days p.i. During the acute and persistent phases of PRRSV infection we could not detect positive cells in heart tissue by ISH (although some viral infectivity was detectable) (Table 1). These results, although contrasting with the results reported by Halbur et al. (15), are not surprising on the basis

of the known diversity of tissue tropism that exists among different PRRSV strains (16).

Ultimate confirmation of the macrophage character of the PRRSV-positive cells by IHC will require the use of different MAbs whose specificity is not impaired by formalin fixation or the use of a different fixation protocol (i.e., cryostat section plus acetone fixation for IHC and PLP fixation for ISH in consecutive sections).

Our study has demonstrated that ISH and IHC both detect PRRSV-infected cells at different times p.i. However, ISH is more sensitive and specific than IHC, especially when studying PRRSV-infected cells at long periods p.i. The acute and persistent phases of infection appear to be based mainly in macrophages. This is consistent with what has been described for the lactate dehydrogenase-elevating virus, the most studied member of the genus *Arterivirus* (4). In view of the results reported here, it is possible that the persistence of PRRSV in vivo involves a continuous level of PRRSV replication in infected cells. With the further use of ISH for transcription-specific studies, we can anticipate that a more subtle molecular analysis of persistent PRRSV infections can be achieved, allowing a better understanding of the pathogenesis of PRRSV.

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