In situ hybridization technique for the detection of swine enteric and respiratory coronaviruses, transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), in formalin-fixed paraffin-embedded tissues

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Accepted 26 June 1995

Abstract

The in situ hybridization (ISH) technique was developed to detect the swine coronaviruses, transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), in cell culture and tissue sections from TGEV-or PRCV-infected pigs. The 35S-labeled RNA probes were generated from two plasmids pPSP.FP1 and pPSP.FP2 containing part of the S gene of TGEV. The procedure was first standardized in cell cultures. The radiolabeled pPSP.FP2 probe detected both TGEV and PRCV in virus-inoculated cell cultures, whereas pPSP.FP1 probe detected TGEV but not PRCV. The probe was then used to detect TGEV or PRCV in tissues of pigs experimentally infected with TGEV or PRCV or naturally infected with TGEV. Again, the probes detected TGEV in intestines of experimentally and naturally infected pigs and PRCV in the lungs of experimentally infected pigs. TGEV RNA was detected mainly within the enterocytes at the tips of villi and, less often, within some crypt epithelial cells. PRCV was shown to replicate mainly in the bronchiolar epithelial cells and in lesser amount in type II pneumocytes, type I pneumocytes, alveolar macrophages and bronchial epithelial cells, respectively. ISH has potential applications as a diagnostic test for the detection and differentiation of TGEV and PRCV in tissues and in studies to gain a better understanding of the mechanism of pathogenesis of enteric and respiratory coronavirus infections.

Keywords: Single stranded RNA probe; In situ hybridization; Transmissible gastroenteritis virus (TGEV); Porcine respiratory coronavirus (PRCV); Coronavirus

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SSDI 0166-0934(95)01901-4
1. Introduction

Coronaviruses are a common cause of enteric and/or respiratory infections in animals and man. Transmissible gastroenteritis virus (TGEV) of swine is one of the best studied coronaviruses, yet diagnostic methods for its detection are less than ideal and limited information is available on mechanisms of TGEV pathogenesis. TGEV causes transmissible gastroenteritis (TGE) in swine characterized by vomiting, severe diarrhea and high mortality in piglets under 2 weeks of age (Bohl and Pensaert, 1989; Saif and Wesley, 1992). In recent years, another coronavirus has been identified in swine and has been designated as porcine respiratory coronavirus (PRCV) which is antigenically closely related to the enteropathogenic TGEV. PRCV was first isolated in 1984 from pigs in Belgium (Pensaert et al., 1986) and is now ubiquitous in swine herds in Europe. PRCV has since been isolated from swine in the US (Hill et al., 1989; Vaughn et al., 1994; Paul et al., 1994). PRCV is different from TGEV in that PRCV has 62 - 68 nucleotide deletion in the 5' end of S gene (Rassachaert et al., 1990; Wesley et al., 1990; Britton and Page, 1991; Wesley et al., 1991a; Paul et al., 1992; Sanchez et al., 1992; Laude et al., 1993; Paul et al., 1994; Vaughn et al., 1994; Vaughn et al., 1995) and deletions in open reading frame 3 (Rassachaert et al., 1990; Britton and Page, 1991; Wesley et al., 1991a). The S gene of TGEV plays an important role for tissue tropism (Parker et al., 1989; Rassachaert et al., 1990; Laude et al., 1993). The deletion in S gene of PRCV causes virus to replicate only in the respiratory tissue rather than in both respiratory and intestinal tissues as for TGEV (O'Toole et al., 1989; Cox et al., 1990). Because PRCV is similar antigenically to TGEV, this creates a diagnostic problem. The most commonly used method for TGEV detection in diagnostic cases is immunofluorescence method on frozen sections of intestines. This technique has many problems as sections or villus enterocytes often wash off the slides during processing. Virus can be isolated in cell cultures, however many virus isolates do not replicate well in cell culture (Vaughn and Paul, 1993). Other methods have been developed such as dot blot hybridization (Shockley et al., 1987; Paul et al., 1994), slot blot hybridization (Wesley et al., 1991b), immunofluorescence (Morrin et al., 1973; Frederick et al., 1976; Shepherd et al., 1979), peroxidase-antiperoxidase staining technique (Chu et al., 1982) and recently immunogold silver staining (IGSS) (Larochelle and Mogar, 1993). All of these techniques have unique advantages and disadvantages and, except for immunohistochemistry, are not suited for detection of virus in tissue sections.

In situ hybridization (ISH) techniques can be utilized to identify specific nucleic acid in cells. Both ISH and immunohistological staining methods have the advantages of requiring only an ordinary microscope and providing a permanent record, and they can be used for retrospective studies and have very high sensitivity and specificity. Therefore, collectively they can provide information on the presence of nucleic acid and antigen and can be used for pathogenesis studies. This study was undertaken to evaluate the ISH technique for the detection of TGEV and PRCV in infected cell cultures, to distinguish TGEV-infected cells from PRCV-infected cells, and to detect TGEV and PRCV in formalin-fixed paraffin-embedded sections from pigs infected experimentally or naturally with TGEV.

2. Materials and methods

2.1. Viruses

The virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, IA) and PRCV strain AR310 were used as reference viruses (Vaughn and Paul, 1993; Vaughn et al., 1994; Vaughn et al., 1995).

2.2. Cell culture

Swine testes (ST) cells were cultured on 8-well chamber slides (Nunc) as reported by Vaughn et al. (1994) by growing in minimum essential medium (Gibco) supplemented with 10% heat inactivated bovine calf serum (Hazelton) and antibiotics. Four wells of 8-well chamber slides were inoculated with 200 plaque-forming units (PFU) of TGEV or PRCV per well. Another four wells served as non-inoculated controls. Twenty hours after inoculation, slides were fixed in 4% buffered paraformaldehyde at 4°C for 1 h.
2.3. Source of tissues

In experiment 1, four 8.5-week-old TGEV- and PRCV-negative pigs were divided into 2 groups. Two pigs were inoculated with $10^5$ PFU of virulent Miller strain of TGEV and two pigs served as non-inoculated controls. In experiment 2, four 5-week-old TGEV and PRCV negative pigs were divided into two groups. Two pigs were inoculated with $10^5$ PFU of PRCV strain AR310 and two pigs served as non-inoculated controls. Tissues from seven pigs submitted as separate diagnostic cases to the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University were also used as naturally-infected pigs and non-infected pigs. Five of the cases had frozen sections diagnosed as TGEV antigen positive and two were diagnosed as TGEV antigen negative by the standard immunofluorescence method. Histopathology on these animals supported the FA results.

2.4. Processing of tissues for in situ hybridization

TGEV inoculated pigs were killed and necropsied at 3 days and PRCV inoculated pigs were killed and necropsied at 4 days post inoculation (DPI). At the times of necropsy, 1 cm segments of the posterior part of duodenum, and the anterior and posterior part of jejunum and ileum from two TGEV-inoculated pigs and 6 pieces of lung from two PRCV-inoculated pigs were collected and fixed in 10% neutral buffered formalin. A random segment of the small intestines of the five pigs infected naturally with TGEV and the two TGEV negative pigs were placed in 10% neutral buffered formalin. The tissues were processed for 48 h in an automated tissue processor and embedded in paraffin. Sections of 5-μm-thickness were cut and placed on nuclease-free silylated slides (PGC Scientific). These slides were deparaffinized and hydrated by incubating at 55°C for 30 min and subsequently passed in absolute xylene 3 times for 5 min, and 1 min each in 3 changes of absolute ethanol, 95% ethanol, 70% ethanol and diethylpyrocarbonate (DEPC, Sigma Chemical Co.) – treated water, respectively.

2.5. Preparation of probes

RNA probes were prepared from plasmid pPSP.FP₁ and pPSP.FP₂ containing the left half of the S gene of TGEV cloned in phagemid vector (Vaughn et al., 1994). The plasmid pPSP.FP₁ contains S gene segment from nucleotides 1678 to 2250 (Britton and Page, 1990) which is present in TGEV but is deleted in all PRCV (Paul et al., 1994; Vaughn et al., 1994). The plasmid pPSP.FP₂ contains S gene segment from nucleotides 1678 (Britton and Page, 1990) to 2483 (Rassachaert and Laude, 1987). The plasmids pPSP.FP₁ and pPSP.FP₂ were linearized with restriction enzymes BamHI and HpaI (Promega), respectively. The in vitro transcription and labeling of the probes were performed as described previously (Gibson and Polak, 1990; Angerer and Angerer, 1992) by using T7-RNA polymerase for plasmid pPSP.FP₁ and T3-RNA polymerase for plasmid pPSP.FP₂ (Promega), the four NTPs (ATP, CTP, TTP and GTP; Sigma Chemical Co.), DTT (dithiothreitol; Bio-Rad) and $[35S]$UTP (Amersham). The transcription reaction was stopped by adding 1 μl (1 μg/μl) of DNase I (Worthington) and 1 μl (1 μg/μl) of RNasin (Promega) for 20 min at 37°C. Unincorporated $[35S]$UTP were removed by passing the labeled RNA through a Sephadex G-25 spin column (Boehringer Mannheim).

2.6. In situ hybridization

Deparaffinized tissue sections and cells in 8-well chamber slides were treated with 2 ml of proteinase K (Ameresco) for 10 min at 37°C in a moist chamber. Concentration of proteinase K was 0.5 μg/ml for cell cultures and 1 μg/ml for tissue sections. Slides were washed in DEPC-treated water (0.1% DEPC) for 1 min. These slides were placed in a staining dish containing 400 ml of 0.1 M triethanolamine (pH 8.0) and 1 ml acetic acid and rapidly stirred for 10 min at room temperature. Slides were prehybridized in 2 × SSC for 5 min and dehydrated in graded alcohols and air dried. The hybridization solution (Brahic and Ozden, 1992; Angerer and Angerer, 1992) containing 50% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate (pH
1 x Denhardt’s solution, 0.1 mg/ml yeast tRNA, 10 mM DTT, and 1 μl of 35S-labeled RNA probe (2 × 10^6 cpm/μl). The amount of hybridization solution added was 50 μl per slide as previously described (Angerer and Angerer, 1992), and coverslips were placed on the slides. Slides were incubated at 52°C for 20 h. The coverslips were removed by soaking the slides in 2 × SSC twice for 5 min each at room temperature. The slides were treated with RNase A (Worthington) (Gibson and Polak, 1990; Angerer and Angerer, 1992; Wilcox, 1993) solution (20 μg/ml) containing 10 mM Tris HCl (pH 8.0) and 0.5 M NaCl for 30 min at 37°C. Slides were washed in 2 × SSC for 5 min at 52°C, 1 × SSC for 5 min at 52°C, 0.5 × SSC for 1 h at 52°C and 0.5 × SSC for 5 min at room temperature. Slides were dehydrated, air dried and were exposed to X-ray films (Fuji Photo film) at −70°C for 24 h. X-ray films were developed and evaluated. The slides were then coated with Kodak NTB-2 emulsion gel (Eastman Kodak Co.) which was diluted with 600 mM ammonium acetate (Kohler and Nelsen, 1990; Angerer and Angerer, 1992) and preheated to 42°C (Gibson and Polak, 1990; Kohler and Nelsen, 1990; Angerer and Angerer, 1992; Wilcox, 1993). These slides were kept in a light-proof box at 4°C for 2 days. The slides were developed at 15°C for 3 min using developer (Kodak D-19) diluted 1:1 with water, followed by a 20 s rinse in deionized water and then fixed for 3 min in fixer. The slides were then rinsed with deionized water for 5 min (Wilcox, 1993) and counterstained with hematoxylin and eosin. All of the solutions used in ISH, except for the RNase A solution to remove contaminating ribonucleases, were prepared by using DEPC-treated water (0.1% DEPC).

Controls included uninoculated ST cell monolayers, RNase-treated cells inoculated with TGEV, tissues from two non-infected animals, RNase A treated tissue sections from TGEV-infected animals, positive strand 35S-labeled RNA hybridized with sections from TGEV-infected animals, and excess amount of non-labeled RNA probe hybridized to sections from TGEV-infected animals followed by hybridization with 35S-labeled RNA probe. The positive strand 35S-labeled RNA probe was generated by using the same plasmid DNA and T7-RNA polymerase (Promega). The negative controls for PRCV inoculation experiment were similar to the TGEV inoculation experiment except RNase A-treated tissue sections from PRCV-inoculated animals and 8-well chamber slides inoculated with PRCV, 35S-labeled RNA probe produced from plasmid pPSP.FP, and the positive strand 35S-labeled probe was generated by using the same plasmid DNA but linearized by using restriction enzyme EcoRI and T3-RNA polymerase.

3. Results

3.1. Detection of TGEV and PRCV in cell cultures

The in situ hybridization technique was first standardized using cell monolayers of ST cells. Uninoculated cell cultures and those inoculated with TGEV or PRCV were hybridized with the RNA probe made from plasmids pPSP.FP, and pPSP.FP,. Following hybridization, slides were exposed to X-ray films. A good dark signal on X-ray films in TGEV or PRCV inoculated wells was usually observed after 24 h exposure and inoculated wells could be differentiated from the uninoculated wells. The virus inoculated wells showed heavy dark squares of silver grains, but non-inoculated wells had only pale gray color as non-specific background. The RNA probe prepared from plasmid pPSP.FP2 detected both TGEV and PRCV whereas the probe prepared from pPSP.FP reacted with TGEV but not PRCV (Fig. 1). The slides were then coated with emulsion gel, stored in the dark, developed after 2 days and examined under the light microscope. A heavy concentration of silver grains localized above and within the cytoplasm of the TGEV or PRCV-infected cells were detected (Fig. 2), only a very low concentration of silver grains were scattered throughout the non-infected cells. Positive signal was abolished by RNase A treatment of cells prior to hybridization as well as by addition of unlabeled RNA probe prior to hybridization with radiolabeled probe.
3.2. *In situ* hybridization of sections of *TGEV*-infected tissues

Once the technique was standardized using cell monolayers, it was tested on intestinal sections from uninoculated and *TGEV*-inoculated pigs. The autoradiography results using X-ray films clearly distinguished between sections from *TGEV*-inoculated and non-inoculated animals as shown in Fig. 3. The sections of duodenum, anterior jejunum, posterior jejunum, anterior ileum and posterior ileum from infected animals gave heavy dark lines of silver grains along the mucosa. No signal was detected in sections from non-infected animals. RNase A-treated sections from infected animals and sections treated with excess amount of non-labeled RNA probe demonstrating the specificity of the probe. Slides coated with emulsion gel from the infected animals had heavy concentration of silver grains along the mucosal layer, but the sections from non-infected animals did not. There were numerous silver grains over the cytoplasm of the villous enterocytes (Fig. 4B). A few non-specific silver grains, which were not localized over the cells, were scattered throughout the sections. Viral RNA was detected in some crypt epithelial cells (Fig. 4D). The histopathologic changes were villous atrophy, blunted and denuded villi and replacement of the columnar cells by flat to cuboidal cells.
Once the technique was standardized on tissues of experimentally infected pigs, we wanted to determine if it could be used on tissue sections from pigs naturally infected with TGEV. We took intestinal sections from five pigs that had been diagnosed as TGE by FA and two that had been TGE negative. As expected, sections from TGE positive pigs were positive by ISH and the TGE-negative sections were negative by ISH.

Fig. 2. In situ hybridization of uninoculated control (A) and TGEV-inoculated ST cells (B) with $^{35}$S-RNA probe produced from plasmid pPSP.FP$_2$ containing TGEV S gene insert. Eight-well chamber slides were exposed to autoradiographic emulsion gel for 2 days.
3.3. In situ hybridization of formalin-fixed paraffin-embedded sections of PRCV infected tissues

Tissues from PRCV-inoculated pigs were used to determine whether this technique could be used to detect PRCV in PRCV-inoculated pigs. The autoradiography results from X-ray films of the lung sections of the PRCV-inoculated pigs and non-inoculated control pigs distinctly illustrated the differences between these two groups of sections. X-ray films of the lung sections from PRCV-inoculated pigs showed the dark ring of silver grains along the small airways and dark spots scattered throughout the sections, but the lung sections from non-inoculated pigs had homogeneous pale gray color over the lung sections. The slides from the PRCV-inoculated pigs coated with emulsion gel had heavy concentration of silver grains localized over the epithelial cells of bronchi, bronchioles, type II pneumocytes, type I pneumocytes and alveolar macrophages. The hybridization signals were also found over the necrotic epithelial cells that sloughed into the lumen of the bronchioles, and over the alveolar macrophages in the exudate in the alveoli and bronchioles. The cells that most often had the localization of the silver grains were bronchiolar epithelial cells (Fig. 5), especially in terminal bronchioles, followed by type II pneumocytes, alveolar macrophages and type I pneumocytes. Only a few bronchial epithelial cells were shown to have localization of the silver grains. The microscopic examination showed mild to moderate necrosis and attenuation of the bronchiolar epithelial cells, moderate lymphomacrophagic infiltration in the alveolar septa, mild type II pneumocyte hypertrophy and proliferation, and lymphomacrophagic exudation in bronchioles and alveoli.

4. Discussion

In this study, an ISH technique is described for the detection and differentiation of coronaviruses TGEV and PRCV in cell cultures as well as formalin-fixed paraffin-embedded tissues of pigs. This test was first standardized in cell cultures. ISH with radiolabeled probes prepared from plasmids pPSP.FP, and pPSP.FP, were highly specific for the detection of TGEV and PRCV in cell culture. The TGEV-infected cells had hybridization signals with radiolabeled RNA probes produced from either of the two plasmids pPSP.FP, and pPSP.FP,. In contrast, the PRCV-infected cells had hybridization signals with radiolabeled RNA probes produced from plasmid pPSP.FP, but not with probes generated from plasmid pPSP.FP,. Non-infected cell cultures did not show hybridization signals with RNA probes generated from plasmid pPSP.FP, or pPSP.FP,. Thus, it was possible to distinguish TGEV-infected cells from PRCV-infected cells by using 35S-RNA probe produced from plasmid pPSP.FP,. This plasmid contained TGEV nucleotides sequences in the S gene that were missing in PRCV (Vaughn et al., 1994). The results from X-ray films corresponded well with the results from emulsion coated slides. For rapid results, slides from suspicious cases can be exposed to X-ray films after hybridization with appropriate 35S-RNA probe. For more details or to differentiate the viruses and to study pathologic changes in tissues, slides can be coated with emulsion gel.

ISH of formalin-fixed paraffin-embedded TGEV-inoculated tissues showed distribution of viral nucleic acid in intestines similar to the distri-
Fig. 4. Photographs of intestinal villi (A and B) and crypt of Lieberkühn (C and D) of small intestines from uninoculated pig (A and C), or pig inoculated with TGEV (B and D) following in situ hybridization with \(^{35}\)S-RNA probe produced from plasmid pPSP.FP. The histopathologic changes observed following TGEV inoculation were villous atrophy, blunted and denuded villi and replacement of the columnar cells by flat to cuboidal cells (B). The ISH signals were specifically localized in villi (B) and over the crypt epithelial cells (D).

bution of viral antigen reported previously (Morin et al., 1973; Frederick et al., 1976; Larson et al., 1979; Woods et al., 1981; Chu et al., 1982; Bohl and Pensaert, 1989; Saif and Wesley, 1992; Larochelle and Mogar, 1993). However, we also detected viral nucleic acid in some crypt epithelial cells. The autoradiography results from X-ray films correlated very well with the results from the emulsion gel coated-slides, and the results were similar in both experimentally inoculated and naturally infected pigs. This result was in contradiction to previous reports where no positive cells were identified in the crypt of Lieberkühn (Morin et al., 1973; Frederick et al., 1976; Shepherd et al., 1979; Larson et al., 1979; Chu et al., 1982; Bohl and Pensaert, 1989; Saif and Wesley, 1992; Larochelle and Mogar, 1993). The reason for this difference is not known except previous studies have examined for viral antigen, whereas, we have looked for viral nucleic acid. TGEV enters into the cell by specific receptors on the cell membrane of mature enterocytes. Two receptors have been identified: aminopeptidase-N (CD 13) (Delmas et al., 1990) and a second putative 200 kD receptor (Weingartl and Derbyshire, 1994). These receptors are found only on the absorptive enterocytes, but they are not found on the cell membrane of the crypt epithelial cells. The mechanism for TGEV infection of crypt epithelial cells is not known. One possibility is that TGEV might bind non-specifically on the cell membrane of the crypt epithelial cells and thus lead to infection of these cells.
Another possibility is that TGEV infection of these cells is non-productive. In this case, there is no viral protein produced in this type of cell, and thus is not detected by immunofluorescence or immunohistochemical methods (Morin et al., 1973; Frederick et al., 1976; Larson et al., 1979; Woods et al., 1981; Chu et al., 1982; Saif and Wesley, 1992; Larochelle and Mogar, 1993). ISH technique also provides for observation of histopathological lesions. The histopathologic changes were similar to previous reports (Morin et al., 1973; Frederick et al., 1976; Larson et al., 1979; Woods et al., 1981; Chu et al., 1982; Bohl and Pensaert, 1989; Saif and Wesley, 1992; Larochelle and Mogar, 1993).

The distribution of PRCV ribonucleic acid was most intense in the epithelial cells of the bronchial epithelium and is compatible with the distribution of viral antigen as reported previously (O'Toole et al., 1989). However our results are incompatible with those observed by Cox et al. (1990) in which the viral replication occurred mainly in the alveolar cells. The microscopic examination showed microscopic changes in the lung tissue as previous reports (O'Toole et al., 1989; Cox et al., 1990; Halbur et al., 1993).

ISH was described for two swine coronaviruses which should be very useful for diagnostic purposes and retrospective studies. This is also the first description of this technique in TGEV and PRCV infection. Development of a non-radioactive labeled probe will make it even more user friendly. We are exploring the potential of using such a probe. This technique also has applications in elucidating mechanisms of pathogenesis of TGEV and PRCV in swine.
Fig. 5. Photographs of formalin-fixed sections from lungs of uninoculated control (A) and PRCV-inoculated (B) pigs hybridized in situ with $^{35}$S-RNA probe produced from plasmid pPSP.FP, and exposed to autoradiographic emulsion for 2 days. ISH signals can be observed over the bronchiolar epithelial cells of small bronchiole of PRCV-inoculated pigs.

Acknowledgements

We thank Dr. Igor Morozov for technical guidance. This study was funded in part with a grant from the Iowa Livestock Health Advisory Council. Theerapol Sirinarumitr is a fellow from the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Kasetsart University, Thailand and is supported with a King's scholarship from the Anandhamahidol Foundation, Thailand.
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