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SHORT PAPER

Detection of Nucleic Acids of Porcine Reproductive and Respiratory Syndrome Virus in the Lungs of Naturally Infected Piglets as Determined by In-situ Hybridization

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Summary

Replication of porcine reproductive and respiratory syndrome virus (PRRSV) was studied in formalin-fixed paraffin wax-embedded lung tissues from seven naturally infected piglets by in-situ hybridization with a non-radioactive digoxigenin-labelled probe. A 433 base pair cDNA probe for the viral RNA encoding the nucleocapsid proteins of a Korean PRRSV isolate was generated by the polymerase chain reaction. All seven piglets infected with PRRSV showed a distinct, positive signal, scattered throughout the alveolar septa and spaces. Positive cells typically exhibited dark brown staining deposits in the cytoplasm without background staining. In-situ hybridization demonstrated that PRRSV replicated primarily in interstitial and alveolar macrophages, and occasionally in type 2 pneumocytes. The bronchial or bronchiolar epithelium did not exhibit a hybridization signal for PRRSV nucleic acids. The anterior and middle lobes of the lung were more reliable than the caudal or accessory lobes for the detection of PRRSV nucleic acids. The in-situ hybridization technique used was rapid, specific and sensitive, and may prove useful for the diagnosis of PRRSV infection in routinely fixed and processed tissues. © 1997 W.B. Saunders Company Limited

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failure in sows, respiratory disease and increased preweaning mortality in sucking pigs, and a mild influenza-like disease in "grower-finisher" pigs (Wensvoort *et al.*, 1991; Collins *et al.*, 1992; Mengeling *et al.*, 1994). A frequent feature of infection with PRRSV is secondary bacterial infection, for example with *Haemophilus parasuis*, *Streptococcus suis*, *Salmonella* spp. or *Actinobacillus pleuropneumoniae* (Dee and Joo, 1994; Mengeling *et al.*, 1994). PRRSV has been characterized and classified in the recently proposed Arterivirus group, with morphological, physicochemical and genetic properties similar to those of the non-arthropod-borne togaviruses, such as lactate dehydrogenase-elevating virus, equine arteritis virus, and simian haemorrhagic fever virus (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993). The genome of PRRSV

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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 ORF7 and is highly conserved among both North American and European isolates (Mardassi *et al.*, 1994; Meulenberg *et al.*, 1995).

In-situ hybridization is a valuable adjunct to standard RNA extraction techniques for evaluating gene expression in tissues and cells. Its major advantage is the ability to determine which cells, in a mixed population, or tissues are expressing the RNA of interest. In experimentally infected pigs, PRRSV nucleic acids were detected by in-situ hybridization in alveolar macrophages and type 2 pneumocytes, and in macrophages from several tissues (Larochelle *et al.*, 1996; Sur *et al.*, 1996). However, only one naturally infected pig was examined (Larochelle *et al.*, 1996). The purpose of the present study was to attempt, by in-situ hybridization, the detection of PRRSV nucleic acids in formalin-fixed paraffin wax-embedded lung tissue from piglets naturally infected with PRRSV, and to demonstrate the diagnostic value of the method.

Materials and Methods

From seven herds, seven piglets naturally infected with PRRSV were killed by electrocution 2 to 4 days after the appearance of clinical symptoms. Tissue samples from four different areas of the right lung (anterior lobe, middle lobe, caudal lobe and accessory lobe) of each pig were fixed in 10% neutral buffered formalin for in-situ hybridization. The left lung was used for PRRSV isolation as described by Halbur *et al.* (1996), and for bacterial isolation. Bacterial cultures were made on 5% defibrinated bovine blood trypticase soy agar, brilliant green agar, and MacConkey agar, incubated at 37°C.

A 433 base pair cDNA fragment representing the 5′ region of the nucleocapsid gene (ORF7) was used as a probe. The polymerase chain reaction (PCR) was carried out as previously described (Mardassi *et al.*, 1994). The sense (101 PLS) and antisense (1011 PLR) primers were 5′-ATGGCCAGCCAGCCAGTCAATCA-3′ (nucleotides 51 to 69) and 5′-TCGCCCTAATTGAATAGGTG-3′ (nucleotides 464 to 483), respectively. The PCR product was purified in agarose gel with commercial geneclean kit (Bio 101 Inc., La Jolla, CA, USA). Purified PCR product was labelled by random priming with digoxigenin-dUTP by means of a commercial kit (Boehringer Mannheim, Indianapolis, IN, USA), according to the manufacturer's instructions.

For in-situ hybridization, lung tissue from each piglet was collected in 10% neutral buffered formalin, and after 1 or 2 days' fixation was dehydrated through graded alcohols and a toluene step and embedded in paraffin wax. Sections were then cut at a thickness of 4 μm, mounted on "Superfrost/plus" slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at room temperature. Just before use, sections were dewaxed in xylene and rehydrated in phosphate-buffered saline (PBS; pH 7·4, 0·01 M) for 5 min. De-proteinization was carried out in 0·2 M HCl for 20 min at room temperature. Tissues were then digested at 37°C for 20 min in proteinase K (Gibco BRL, Grand Island, NY, USA) 20 μg/ml in PBS (pH 7·4, 0·01 M) and fixed in paraformaldehyde 4% 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%) had been added. After 5 min, a further 0·75 ml of acetic anhydride was added and 5 min later the slides were rinsed in 2 × saline sodium citrate (SSC) (1 × SSC contains 50 mM NaCl and 15 mM sodium citrate pH 7·0). The slides were equilibrated for 60 min in a standard hybridization buffer that consisted of 5 × SSC containing deionized formamide 50%, 10 × blocking buffered solution (Boehringer Mannheim) 2%, N-lauroylsarcosine 0·1%, and sodium dodecyl sulphate (SDS) 0·02%.

Table 1
Microbiological and in-situ hybridization results in seven piglets naturally infected with PRRSV

Piglet no.	*Age (days)*	*Bacterial isolates*	*In-situ hybridization results*		
			Interstitial macrophages	*Alveolar macrophages*	*Type 2 pneumocytes*
1	7	None	+++	+	+
2	6	None	++	+	−
3	13	*H. parasuis*	++	+	−
4	10	*H. parasuis*	++	++	−
5	5	None	++	+	−
6	7	None	++	+	+
7	12	None	++	+	−

Reaction: −, none; +, slight (<1 cell/high power; ×400); ++, moderate (approximately 1 cell/high power field); +++, extensive (>1 cell/high power field).

Hybridization was carried out overnight at 45°C. The digoxigenin-labelled probe (0·1 ng/μl) was diluted in 300 μl of the standard hybridization buffer and then heated for 5 min in a 95°C heating block and quenched on ice before being applied to tissue sections. Approximately 75 ng of digoxigenin-labelled probe were added to standard hybridization buffer (70 μl), which was then layered over the section. The fluid was held in place by a coverslip, the edges of which were sealed with rubber cement. After overnight hybridization, sections were thoroughly 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 formamide 60% 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 maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7·5) for 5 min at room temperature.

For detection of hybridization, sections were incubated with anti-digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim) diluted 1 in 200 in 0·1 M Tris-HCl (pH 7·4), 0·15 M NaCl with 1% blocking reagent (Boehringer Mannheim). After three washes in buffer, substrate consisting of nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolylphosphate (BCIP) was layered over the sections. Colour was allowed to develop for 5–8 h in the dark, and development was stopped by dipping slides briefly in Tris-ethylenediaminetetraacetic acid buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8·0). Sections were counterstained with methyl green 0·5%, and the slides were then washed with distilled water for 1 min and dried completely. Negative control sections were prepared from a 1-day-old colostrum-deprived pig not exposed to PRRSV. Also included as a control was lung tissue from a 3-week-old conventional pig infected with porcine respiratory coronavirus (PRCV), isolate SK2736.

Results

Clinical history, microbiological and in-situ hybridization results for the seven infected piglets are presented in Table 1. PRRSV was isolated from the lungs of all piglets, and two (nos 3 and 4) were also infected with *Haemophilus parasuis*. It is not known whether piglet no. 6 had a bacterial infection, as it had been treated with antibiotic. Absence of pulmonary mycoplasmosis was inferred from the absence of typical lesions but was not confirmed by culture.

By in-situ hybridization, a distinct, positive signal, scattered throughout the alveolar septa and spaces, was detected in all seven piglets. The signal intensity

varied within and between histological structures in any one section and between piglets. Positive cells typically exhibited dark brown reaction product in the cytoplasm, without background staining. A strong hybridization signal was detected in the cytoplasm of mononuclear cells randomly scattered in the thickened alveolar septa (Fig. 1). A less intense signal was detected on rare occasions in cells that resembled type 2 pneumocytes (Fig. 2). The positive cells generally had large oval nuclei and abundant cytoplasm. Comparison with haematoxylin and eosin-stained sections from the same block indicated that most of the positive cells were interstitial macrophages. The alveolar macrophages frequently showed a strong hybridization signal, representing PRRSV replication (Fig. 3). The distribution of positive cells in the lung was very patchy. PRRSV nucleic acids were detected within the cytoplasm of alveolar macrophages. When hybridization signal was detected in alveolar macrophages, it was usually associated with inflammation. Hybridization signal was occasionally detected in necrotic cellular debris in the lumina of terminal bronchioles (Fig. 4). No hybridization signal was detected in bronchial or bronchiolar epithelium. PRRSV nucleic acids were detected in the anterior and middle lobes of the lungs in six of the seven piglets, but in the accessory and caudal lobes of only four and two, respectively. Sections from the two control pigs showed no hybridization signal.

Discussion

In this study the replication of PRRSV in seven naturally infected piglets was demonstrated by in-situ hybridization with a non-radioactive digoxigenin-labelled probe. PRRSV apparently replicated primarily within interstitial macrophages, alveolar macrophages, and type 2 pneumocytes. No attempt other than by visual examination was made to identify the cell types and our results may need to be confirmed by electron microscopy or other methods of identification.

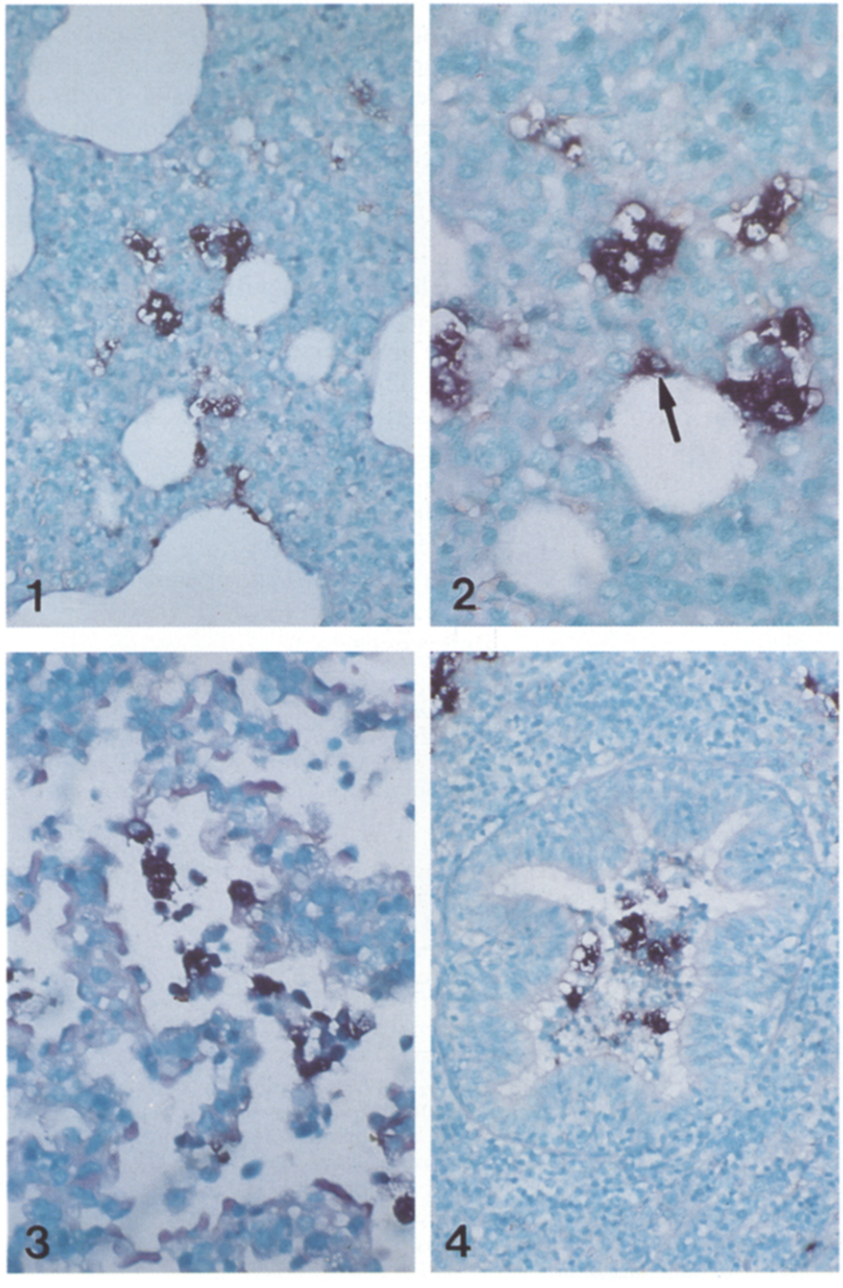
Immunohistochemical studies suggested that PRRSV replicates primarily in macrophages in the lungs (Magar *et al.*, 1993; Halbur *et al.*, 1994, 1996). Destruction of pulmonary macrophages by replicating PRRSV is an indication of a direct pathogenic effect of the virus. Such changes affect adversely the antibacterial defence of the lung and probably predispose to common secondary

Fig. 1. Lung of piglet no. 1. Naturally infected with porcine reproductive and respiratory syndrome virus (PRRSV). In-situ hybridization with a PRRSV probe. Positive hybridization signals for PRRSV nucleic acids occur within the cytoplasm of mononuclear cells in the thickened alveolar septa. Hybridization signals are seen as black grains. Methyl green counterstain. × 200.

Fig. 2. Lung of piglet no. 1. In-situ hybridization with a PRRSV probe. Type 2 pneumocytes contain replicating PRRSV (arrow). Hybridization signals are seen as black grains. Methyl green counterstain. × 400.

Fig. 3. Lung of piglet no. 4. In-situ hybridization with a PRRSV probe. Alveolar macrophages contain replicating PRRSV. Hybridization signals are seen as black grains. Methyl green counterstain. × 200.

Fig. 4. Lung of piglet no. 5. In-situ hybridization with a PRRSV probe. Positive signals occur in cellular debris in terminal airway lumina. Hybridization signals are seen as black grains. Methyl green counterstain. × 200.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bacterial infections, as seen in piglets no. 3 and 4. Secondary bacterial infections are commonly reported in PRRSV-infected pigs (Dee and Joo, 1994; Mengeling *et al.*, 1994). The possible role of other respiratory cells, such as type 2 pneumocytes, in the replication of PRRSV in the lung has been previously demonstrated by immunohistochemical methods (Magar *et al.*, 1993; Halbur *et al.*, 1994). PRRSV nucleic acids or antigens have been detected in type 2 pneumocytes in formalin-fixed sections of lungs by in-situ hybridization (Sur *et al.*, 1996) and in frozen sections of lungs by indirect immunofluorescence (Pol *et al.*, 1991). Our study further confirmed replication of PRRSV in the lung. We were unable to detect PRRSV nucleic acids in bronchiolar epithelium.

The use of non-radioactive in-situ hybridization to study infectious diseases is one of the most promising applications of this technique (Crabb *et al.*, 1992). It appears to be universally applicable and is capable of detecting minute amounts of viral nucleic acids. To avoid non-specific hybridization of the oligonucleotide primers, both reverse transcription and the PCR must be performed under stringent conditions. The in-situ hybridization used in this study should prove valuable for the diagnosis of PRRSV from routinely fixed and processed tissues.

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