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Immunohistochemical Identification of Porcine Respiratory Coronavirus Antigen in the Lung of Conventional Pigs

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Abstract. Nine 3-week-old conventional pigs were inoculated intranasally with a Korean isolate of porcine respiratory coronavirus (PRCV). Three 3-week-old conventional pigs were kept as noninoculated controls. Three inoculated and one control pig were euthanized at 5, 10, and 15 days postinoculation (DPI), respectively. All nine inoculated pigs developed respiratory disease. Interstitial pneumonia characterized by mononuclear inflammatory cell infiltration into alveolar septa were seen microscopically in all nine PRCV-infected pigs. Histopathologic changes were severe at 5 DPI, modest at 10 DPI, and almost resolved at 15 DPI. PRCV antigen was not detected in bronchial and bronchiolar epithelium but was detected in interstitial macrophages in the lungs. This Korean isolate of PRCV appeared to replicate primarily within alveolar macrophages in the respiratory system of the pig.

Key words: Pigs; pneumonia; porcine respiratory coronavirus; respiratory virus.

Porcine respiratory coronavirus (PRCV) was first isolated in Belgium in 1984 and is antigenitically related to transmissible gastroenteritis virus (TGEV).[9] PRCV has since been isolated in North America.[5,15] Nucleotide sequences show slight differences between European and US PRCV isolates. The European PRCV isolates have a 672-nucleotide deletion in the 5′ end of the *S* gene, whereas the US PRCV isolates have a 681-nucleotide deletion in the 5′ end of the *S* gene.[1,10] Although closely related antigenitically, PRCV and TGEV have several striking differences. In contrast to TGEV, PRCV isolates have a deletion in the region that encodes mRNA 3.[10,13] TGEV replicates in both the intestinal tract and respiratory tract without causing respiratory disorder. In contrast, PRCV replicates to high titers in lung tissue and little or not all in the intestinal tissues and with no evidence of villus atrophy or gastroenteritis.[2,8]

It is still debatable whether or not PRCV causes clinical signs. Some field reports have linked a PRCV infection with respiratory disease in growing, finishing, and adult swine. But other natural infections have not caused respiratory signs.[7] Experimentally induced PRCV infections of feeder and fattening pigs have been associated with clinical signs such as dyspnea and polypnea during infection in some studies,[6] whereas in other studies experimental infection did not cause clinical disease.[4,15] We report here on the immunohistochemical identification of PRCV antigen in porcine lung experimentally infected with PRCV.

The anterior nasal cavity of a pregnant sow selected from

a minimal disease herd was swabbed and cultured for PRCV as previously described.[15] No PRCV was isolated from the nasal cavity, and virus neutralization test[6] did not detect antibodies against PRCV or TGEV in the sow's serum. Serum of 12 pigs was tested for antibodies against PRCV and TGEV before experimental infection with PRCV. The pigs were seronegative with respect to PRCV and TGEV.

Nine 3-week-old PRCV- and TGEV-negative conventional Landrace piglets were inoculated intranasally with 1 ml of tissue culture fluid containing 10^7 tissue culture infective doses 50/ml of a Korean isolate of PRCV strain SK2736, propagated on primary swine testicular cells.[5] The inoculum was prepared as effluent from the tissue cultures. Three PRCV- and TGEV-negative control pigs received equal volumes of Hanks' medium by the same routes. These pigs were housed on elevated woven-wire decks and fed a commerical milk replacer. The pigs were monitored for enteric and respiratory signs three times each day. Three PRCV-inoculated and one control pig were euthanatized by electrocution at 5, 10, and 15 days postinoculation (DPI), respectively, and necropsied. Virus isolation was attempted from the lung and intestine as previously described.[5] The methods of procurement, animal facilities, and experimental design were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

For immunohistochemical evaluation, lung and jejunum tissues from each pig were collected in 10% formalin and embedded in paraffin. Tissues were routinely processed, sectioned at 4 μm, and placed on poly-L-lysine-coated glass microscope slides. Tissues were deparaffinized with xylene, rehydrated through graded alcohols, and air dried. Endogenous peroxidase was quenched with absolute methanol containing 1% hydrogen peroxide for 1 hour. All slides were then treated with 0.01% protease (Protease XXIV, Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) for 2 minutes at room temperature. All slides were incubated with 10% normal horse serum (NHS) (Vector Laboratories, Burlingame, CA) and 3% bovine serum albumin mixture (ratio 1:20) in PBS (0.1 M, pH 7.4) for 1 hour at room temperature to saturate nonspecific protein-binding sites. A monoclonal antibody (provided by Dr. T. W. Molitor, University of Minnesota, St. Paul, MN) was diluted 1:800 in PBS containing 2% NHS and 0.3% Triton-X (Sigma Chemical Co.). This monoclonal antibody reacted with 30-kd of a membrane protein of PRCV by western blotting analysis and cross-reacted with TGEV (T. W. Molitor, personal communication). The slides were incubated overnight at 4 C in a humidity chamber.

Following three washes with PBS (0.1 M, pH 7.4), sections were flooded and incubated for 1.5 hours at room temperature with biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:400 in PBS (0.1 M, pH 7.4) containing 2% NHS and 0.3% Triton-X. The slides were washed in PBS (0.1 M, pH 7.4), followed by incubation in avidin–biotin complex (ABC) solution prepared according to the manufacturer's instruction (Vectastain Elite ABC kit, Vector Laboratories) for 30 minutes. After washing in PBS (0.1 M, pH 7.4), the final reaction product was produced by immersing the sections in a solution of 0.01% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories) in PBS (0.1 M, pH 7.4) for 10 minutes. The sections were lightly counterstained with Mayer's hematoxylin, dehydrated through graded concentrations of ethanol and xylene, and mounted. Negative control sections were from a control 1-day-old colostrum-deprived pig not exposed to PRCV and TGEV and from pigs that had been infected with porcine reproductive and respiratory syndrome virus.

Only three PRCV-infected pigs show mild dyspnea at 2 DPI. Six PRCV-infected and three uninfected control pigs remained clinically healthy over the 15 days of the study. Gross lesions were limited to the lungs. At 5 and 10 DPI, all six PRCV-infected pigs had gross pneumonic lesions characterized by reddish tan consolidated areas forming a sublobular or lobular mosaic in the dorsal portion of the cranial and middle lobes. Lesions were slightly more pronounced at 10 DPI. At 15 DPI, there were no visible gross lesions in the infected pigs. No gross lesions were present in control pigs. PRCV was isolated from lung from all virus-inoculated pigs at 5, 10, and 15 DPI. No PRCV was isolated from intestinal samples from virus-inoculated pigs at 5, 10, or 15 DPI. PRCV was not recovered from any control pigs.

Lungs, trachea, lymph node, spleen, kidneys, brain, and small intestine were examined histologically. Significant histopathologic changes were limited to the lungs. Histologic lesions consisted of multifocal interstitial pneumonia characterized by infiltration of a modest number of mononuclear inflammatory cells into alveolar septa. The pulmonary lesions were severe at 5 DPI, moderate at 10 DPI, and nearly resolved at 15 DPI. No significant microscopic changes were observed in lungs or intestines of control pigs. PRCV antigen was detected in lung tissue only. In the lung, antigen was strongly detected in the cytoplasm of mononuclear cells randomly scattered in the thickened alveolar septa of the PRCV-infected pigs. The positive cells generally had large oval nuclei and abundant cytoplasm (Fig. 1). Antigen was most pronounced at 5 DPI. Antigen was not detected in either nucleus or cytoplasm of the bronchial and bronchiolar epithelium. PRCV antigen was not detected in jejunal enterocytes. Comparison with hematoxylin and eosin-stained sections from the same block indicated that most of the positive cells were interstitial macrophages. No PRCV antigen was detected in any tissue of the negative control pigs. Background staining in all specimens was negative or faint.

This study clearly demonstrated that PRCV administered to 3-week-old conventional pigs by intranasal inoculation infected the lungs and produced significant morphologic lesions. In addition, a monoclonal antibody against PRCV could be used to detect PRCV infection in formalin-fixed, paraffin-embedded lung of infected pigs. The ABC immunostaining technique was chosen because it is useful for detecting antigens that are partially denatured by formalin fixation, and it is commonly used for detection of infectious disease agents.[3]

The gross and microscopic lesions that developed in 3-week-old conventional pigs in this study were similar to lesions reported for experimental PRCV infection in pigs.[2,4,5,8,11] Other PRCV isolates replicate extensively in epithelial cells of the upper and lower respiratory tract and in alveolar macrophages.[2,8,9] However, we were unable to detect

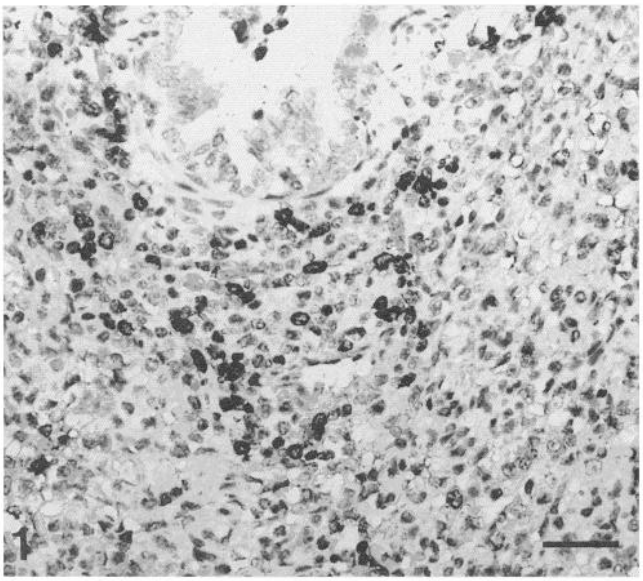

Fig. 1. Lung; pig, 5 DPI. Positive staining for PRCV antigen demonstrated within the cytoplasm of mononuclear cells in the thickened alveolar septa. ABC, H_2O_2, DAB, Mayer's hematoxylin counterstain. Bar = 50 μm.

antigen in epithelial cells of the upper and lower respiratory tract. This Korean isolate of PRCV apparently replicates primarily within interstitial macrophages in the respiratory system of the pig. However, the PRCV antigen staining could represent phagocytized antigen, possibly from infected epithelial cells sloughed during earlier stages of the infection. Additional studies utilizing more pigs necropsied at more frequent intervals and following the antigen localization by immunohistochemical staining in the early stages of disease are necessary. The use of immunohistochemical methods for the detection of viral antigens in formalin-fixed paraffin-embedded tissues offers several advantages over histologic diagnosis. Retrospective diagnoses can be also made because antigens are usually stable indefinitely in paraffin-embedded tissues.

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