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Immunohistochemical identification of porcine reproductive and respiratory syndrome virus antigen in the lungs of naturally infected piglets

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돼지 생식기 호흡기 증후군 바이러스에 자연감염된 포유자돈의 폐장에서 면역조직화학법을 이용한 바이러스 항원의 확인

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초 록 : 돼지 생식기 호흡기 증후군 바이러스의 nucleocapsid와 반응을 하는 SDOW17 단크론항체를 이용하여 중성 포르말린에 고정시킨 자연감염된 포유자돈의 폐장에서 면역조직화학법을 이용하여 돼지 생식기 호흡기 증후군 바이러스 항원을 확인하였다. 서울대학교 수의과대학 병리학교실에 의뢰된 포유자돈들 중에서 병리조직학적으로 폐장에서 간질성 폐렴이 관찰된 포유자돈 7두를 임의로 선택하여 본 실험을 실시하였다. 간질성 폐렴의 병변으로 많은 수의 대식세포 침윤을 동반한 폐포벽 두께의 증가와 제 II 형 폐포세포의 비후가 관찰되었다. 검사한 7두 포유자돈중에서 6두에서 돼지 생식기 호흡기 증후군 바이러스에 대한 항체를 enzyme-linked immunosorbent assay에 의해 확인하였다. SDOW17 단크론항체를 이용한 면역조직화학염색과 간질성 폐렴의 대식세포에서 돼지 생식기 호흡기 증후군 바이러스의 항원을 검출하였고, 항원은 (주로)대식세포의 세포질에서만 진한 갈색의 양성반응이 관찰되었다. 이상 검사결과 돼지 생식기 호흡기 증후군 바이러스는 폐장의 간질과 폐포강에 분포되어 있는 대식세포에서 주로 증식하는 것으로 판명되었다. 본 실험에서 사용한 면역조직화학법은 돼지 생식기 호흡기 증후군 바이러스 감염여부를 바이러스 분리 또는 혈청검사 없이 진단하는데 사용할 수 있는 유용한 진단방법으로 판명되었다.

Key words : immunohistochemistry, interstitial pneumonia, pig, porcine respiratory and reproductive syndrome, togavirus.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first observed in the United States in 1987[8], in Europe in 1990[15], and in the Republic of Korea in 1994.[9] The disease is caused by a virus referred to as the ATCC VR-2332 strain of PRRS virus in the North America[3] and as the Lelystad virus in Europe[20]. The etiologic agent of PRRS is small, enveloped RNA virus with morphologic, physiochemical, and genetic properties similar to those of the non-arthropod-borne togaviruses such as lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus[2,4].

PRRS virus causes reproductive failure in sows, respiratory disease and increased preweaning mortality in suckling pigs and a mild flu-like disease in grower-finisher pigs.[8,20] Clinical signs in PRRS virus-infected pigs are inappetence, fever, diarrhea, dyspnea and rough hair coats.[3] One feature of infection with PRRS virus often described is an increased incidence of bacterial disease, including secondary infection with *Haemophilus parasuis*, *Stereptococcus suis*, *Salmonella* spp., *Pasteurella multocida*, or *Actinobacillus pleuropneumoniae*.[13]

An immunoperoxidase test on formalin-fixed tissues from infected pigs would be useful as a simple diagnostic test and as a mean to study the pathogenesis of PRRSV-induced disease. PRRS viral antigen has been detected by immunohistochemistry in alveolar macrophage in the lung, in endothelial cells and macrophages in the heart, and in macrophages and dendritic-like cell in the lymphoid tissue from experimentally PRRS virus-infected pigs[5,6,10]. In this paper, we describe the avidin-biotin-peroxidase(ABC) complex method, using a monoclonal antibody-based immunoperoxidase test, for localization of PRRS virus in paraffin-embedded lung from naturally PRRS virus-infected preweaning piglets.

Materials and Methods

Sample collections : Lung samples used in this study were selected on the basis of interstitial pneumonia by histopathological examination and serologic positive by enzyme-linked immunosorbent assay. Seven hog farms with a clinical history of PRRS are submitted for diagnosing PRRS virus infection into Department of Veterinary Pathology in Seoul National University. One 250-sow, continuously farrowing swine unit from Chungcheung Nam Do experienced a respiratory problems in 2- to 28-day-old Landrace piglets. Approximately 20% of the piglets in 16 of 20 litters had severe thumping during a 2-week period; mortality was 25%. A few litters of piglets failed to gain weight and were stunted. One 7-days-old Landrace piglet was submitted for diagnosis. The other 300-sow, continuously farrowing swine unit from Kyounggi Do experienced same respiratory problems in preweaning piglets. Respiratory signs are more pronounced in piglets under 3 weeks of ages. Four 2-weeks-old Landrace piglets were submitted for diagnosing respiratory problem. The rest of 5 hog farms had similar respiratory problems.

Enzyme-linked immunosorbent assay : Serum samples were tested for antibodies to PRRS virus using commercial ELISA kit (HerdChek® : PRRS, IDEXX Laboratories, Inc., Westbrook ME, USA) as described by manufacturer. This commercial ELISA test kit is recognized both the American and European strains. Blood samples were collected from jugular vein in each piglet. Blood was allowed to clot, centrifuge and the serum kept at -20℃ until used. One hundred microliter of the 1 : 40 dilution of test sera were added to duplicate well coat with proprietary PRRS viral antigen or normal cell antigen, and incubate for 30 minutes at room temperature. After washing the wells with a phosphate-buffered wash solution containing Tween (300μl/well), the plate were reincubates for 30 minutes and after being washed again, were incubated for 15 minutes with 100μl of TMB (3, 3'5,5'-tetramethylbenzidine) substrate solution. The reaction was then stopped by addition of 100μl of a stop solution containing hydrofluoric acid into each well. Optical density (OD) of reach well was measured at 630nm wave length using ELISA microplate reader. The presence or absence of antibody to PRRS virus was determined by calculating the test sera to positive (S/P) ratio:

S/P = (Δ OD of test serum between viral and control antigen) ÷

(Δ OD of positive reference between viral and control antigen).

Samples were considered to be positive for PRRS virus antibody if the S/P ratio was greater than 0.4.

Immunohistochemistry : For immunohistochemical evaluation, lung tissues from a pig were collected in 10% formalin and embedded in the paraffin. Tissues were sectioned, and placed on poly-L-lysine-coated glass microscope slides. Sections were deparaffinized 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, USA) in PBS (0.1M, pH 7.4) for 2 minutes at room temperature. All slides were blocked with 10% normal horse serum (NHS) (Vector Laboratories, Burlingame, CA, USA) and 3% bovine serum albumin mixture (ratio 1:20) in PBS for 1 hour at room temperature to saturate nonspecific protein-binding sites. The primary antibody used was a monoclonal ascites fluid (SDOW-17) diluted 1 : 500 in PBS containing 2% NHS and 0.3% Triton X (Sigma Chemical Co, St Louis, MO, USA). This monoclonal antibody specifically recognized a conserved epitope on the putative 15 kDa nucleocapsid protein of United States and European isolates of PRRS virus.[14] The slides were incubated overnight at 4℃ in a humidity chamber.

Following three times washing by PBS, sections were incubated for 1.5 hours at room temperature flooded with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 400 in PBS containing 2% NHS and 0.3% Triton X. The slides were washed in PBS, followed by incubation in ABC solution prepared according to the manufacturer's instruction (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes. After washed in PBS, 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) in PBS for 10 minutes. The sections were lightly counterstained with Mayer's hematoxylin, dehydrated through graded concentrations of ethanol and cless in xylene, and mounted. Lung from 1-day-old colostrum-deprived pig was used as negative control, and pig that had been experimentally infected with porcine respiratory coronavirus.

Results

Serology : Results of ELISA for 7 sera are shown in Table 1. Of the 7 sera samples examined for anti-PRRS antibody, 6 serum samples were positive and one serum sample (pig no. 7) was negative by ELISA.

Table 1. Comparison of results of serology, histopathologic pulmonary lesions, and PRRS virus antigen from 7 naturally PRRS virus-infected piglets.

Pig No.	Age(days)	ELISA	Interstitial pneumonia	PRRS virus antigen
1	5	positive[a)]	+[b)]	+[c)]
2	7	positive	+	-
3	8	positive	+	+
4	10	positive	+	+
5	14	positive	+	+
6	14	positive	+	+
7	21	negative	+	+

a) : Antibody against PRRS virus was tested by enzyme-linked immunosorbent assay.
b) : Interstitial pneumonia was observed by light microscope.
c) : PRRS virus antigen was detected in either interstitial or alveolar macrophages by immunohistochemistry.

Gross lesions : Gross lesions of lungs were similar and incosistent in 7 inferted piglets. The ventral one-third of the cranial lung lobes and the ventral one-fourth of the middle lung lobes were purple, soft, and wet in 5-day-old (pig no. 1), 7-day-old (pig no. 2) and 8-day-old (pig no. 3) piglet. There were similar changes in the ventral cranial and ventral middle lung lobes. The discoloration in lung lobes disappeared when they were perfused with formalin. Gross lesions from 10-day-old (pig no. 4) and 21-day-old (pig no. 7) piglet were characterized by multifocal, tan-mottled area, with irregular and indistinct borders in the cranial and accessory lobes. Two 14-day-old piglets (pig nos. 5 and 6) had tan consolidation in all of the left apical and 50% of the left middle lung lobes.

Histopathology : Microscopic lesions were consistently found in the lung of naturally infected piglets. All infected piglets had interstitial pneumonia. Cranial lung lobes had

the most severe and frequent lesions. The pneumonic lesions were multifocal (pig no. 1, 2, 3 and 4), mild (pig no. 7) to moderate (pig no. 5 and 6). In the pig no. 1, 2, 3 and 4, the interstitial pneumonia was characterized by three main changes: i) infiltration of mononuclear cells at alveolar septa, ii) pronounced type 2 pneumocyte hypertrophy and hyperplasia, and iii) abundant accumulation of necrotic cells debris and mixed inflammatory cells in the alveolar spaces. In two 14-day-old (pig no. 5 and 6) and one 21-days-old (pig no. 7) piglet, the interstitial pneumonia was characterized mainly by septal thickening with mononuclear cells, peribronchiolar and perivascular lymphohistiocytic cuffing, and accumulation of macrophages and necrotic debris in alveolar spaces. Type 2 pneumocyte hyperplasia and hypertrophy were less consistent and less severe than in the pig nos. 1, 2, 3 and 4.

Immunohistochemistry : Immunohistochemically, presence of PRRS virus antigen correlated closely with the interstitial pneumonia in the lungs. Overall, 7 lungs except one (pig no. 2) that had microscopic lesions of PRRS stained positively for PRRS virus antigen. Staining of the PRRS virus antigen varied from no staining to very strong staining, often within a single cluster. There was multifocal staining of granular cytoplasm in moderate numbers of positive cells infiltrated into thickened alveolar septa (Fig 1, 2). PRRS virus-antigen-positive cells were concentrated in the center of peribronchiolar lymphoid tissue. Intense and specific celluar cytoplasmic staining was observed in the peribronchiolar lymphoid tissue (Fig 3). Lesser numbers of PRRS virus-antigen-positive oval cells resembling small macrophages were observed in the peribronchiolar lymphoid tissue (Fig 3). Occasionally, PRRS virus-antigen-positive cells were focally seen in the perivascular area (Fig 4). There were no false positive staining in any of the 5 control lungs from same aged piglets. Background staining in all specimens was negative or faint.

Discussion

This study demonstrated that a monoclonal antibody against a PRRS virus could be used to detect PRRS virus in formalin-fixed, paraffin-embedded lung of infected piglets. Comparison with hematoxylin and eosin-stained sections from the same block indicated that most of the labeled cells were macrophages, and some were likely sloughed pneumocytes. Because PRRS virus replicated *in vitro* and most of the labeled cells were macrophages by ABC method, we and other researcher[5] strongly suggested that PRRS virus is primarily replicated in the pulmonary alveolar macrophage. PRRS viral antigens were detected in epithelial cells of bronchioles and alveolar ducts and within cells in the alveolar septa and alveolar space on frozen sections.[16] However, we and other researcher[5] were unable to detect antigen in bronchiolar epithelium.

Our results suggested that naturally PRRS virus-infected pneumonic lesions develop subsequent to viremia and are consistent with interstitial pneumonia as experimentally PRRS virus-infected pigs.[3,7,18,19] Viremia may help PRRS virus dissemination to several tissues such as heart, lymph node and brain. PRRS virus has recently been shown by immunohistochemistry to replicate in dendritic-like cells in the lymphoid system and endothelial cells and macrophages in the heart of experimentally PRRS virus-infected pigs.[6] In addition, the PRRS virus can be isolated from the brain, lymph node, spleen, tonsil and serum from experimentally PRRS virus-infected pigs.[3,7,18,19]

Only one pig which had viremia did not have immunologic reactivity. There are several possible causes of negative immunoreactivity.[4] Formalin fixation results in some degree of antigen denaturation due to cross-linking of amino groups.[17] The maximal time tissue should remain in formalin for optinum immunoreactivity may be less than 24 hours. Our tissue may often remained in formalin for more than 24 hours that decreased immunoreactivity. 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.[4]

PRRS virus infection was diagnosed on the basis of histopathology, immunohistochemistry, serology and virus isolation.[1,5~7,10,11,21] The histopathological diagnosis of infection by PRRS virus is limited since other viral pathogens may in-

duce similar lesions. The isolation and identification of PRRS virus is complex and labor intensive and may require many days or weeks to perform. The primary advantages of using an immunohistochemical technique to detect viral antigens in formalin-fixed specimens, rather than attempting virus isolation from fresh tissues, are save the time and expense for rendering a definitive diagnoses and the ability to associate the virus with specific cells, and to perform retrospective studies on paraffin-embedded tissue since viral antigens are usually stable indefinitely in paraffin-embedded tissue.

In summary, the immunohistochemical staining method described in this report is highly specific and rapid detective for PRRS virus infection with no cross-reactivity of other common porcine respiratory infectious agent such as porcine respiratory coronavirus. Further studies using immunohistochemical techniques should provide a better understanding of the pathogenesis of PRRS virus infection in pig.

Conclusion

Porcine reproductive and respiratory syndrome (PRRS) virus antigen was detected by an immunohistochemical technique in lung tissues of 7 preweaning piglets showing respiratory syndromes that has been infected naturally with PRRS virus. Of the 7 serum samples examined for anti-PRRS virus antibody, 6 were positive and one was negative by enzyme-linked immunosorbent assay. PRRS virus antigen was readily detected in alveolar macrophages in the formalin-fixed paraffin-embedded lungs from natural infected with PRRS virus. The labeled antigen was primarily within the cytoplasm of sloughed cells and macrophages in the alveolar spaces and septae. Immunohistochemical staining of formalin-fixed lungs from preweaning piglets with interstitial pneumonia was helpful as a routine diagnostic test for substantiating a diagnosis of PRRS virus infection.

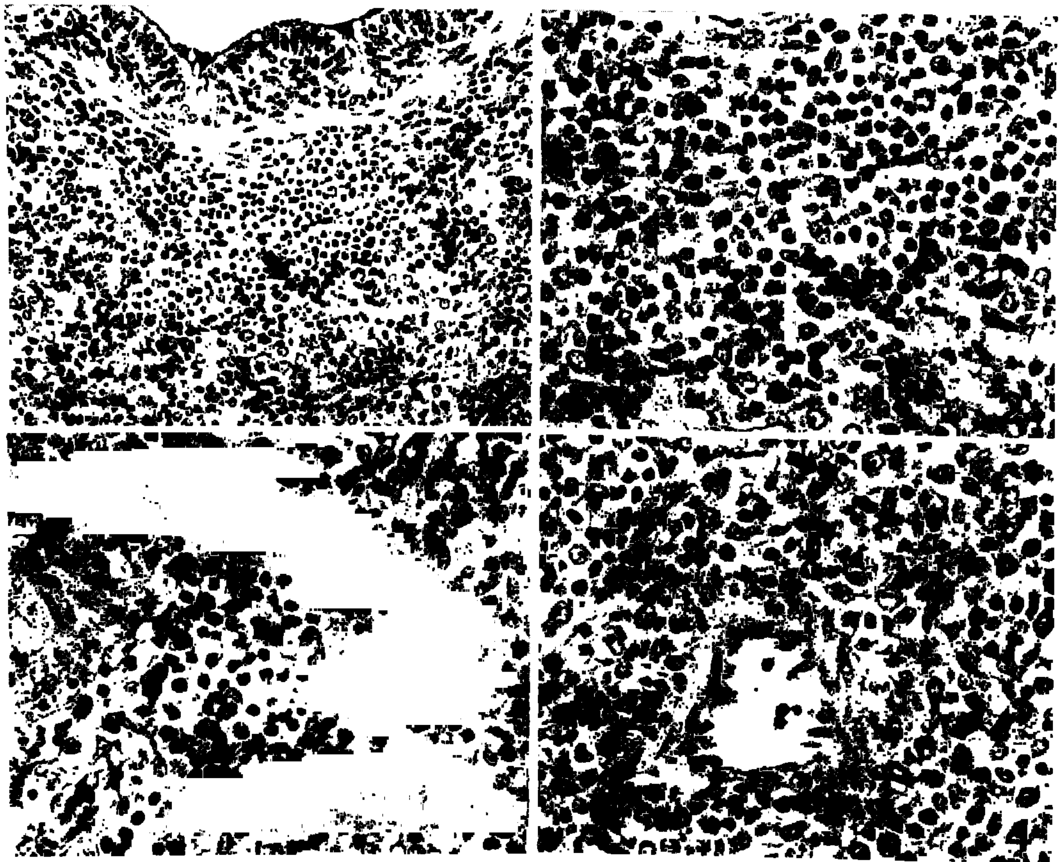
Legends for figures

Fig 1. Lung from a 5-day-old piglet (pig no. 1). PRRSV-antigen-positive cells (arrow) within thickened alveolar septa. Avidin-biotin complex method, Mayer's hematoxylin counterstain, × 200.

Fig 2. Lung from a 5-day-old piglet (pig no. 1). PRRSV-antigen-positive cells (arrow) within thickened alveolar septa. Avidin-biotin complex method, Mayer's hematoxylin counterstain, × 400.

Fig 3. Lung from a 8-day-old piglet (pig no. 3). PRRSV-antigen-positive cells (arrow) in the peribronchiolar lymphoid tissue. Avidin-biotin complex method, Mayer's hematoxylin counterstain, × 400.

Fig 4. Lung from a 21-day-old piglet (pig no. 7). PRRSV-antigen-positive cells (arrow) in the perivascular area. Avidin-biotin complex method, Mayer's hematoxylin counterstain, × 400.



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