Immunohistological Evaluation on Respiratory Lesions of Pigs Intrasasally Inoculated with Actinobacillus pleuropneumoniae Serotype 1

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ABSTRACT. Nine-week-old pigs were inoculated intranasally with 6.10 × 10⁶ group 10⁵, 10⁴ (group 10⁴) and 10³ (group 10³) colony-forming unit of Actinobacillus pleuropneumoniae (App) serotype 1 designated HA-337 strain, respectively. One pig in group 10⁶ and 2 pigs in group 10⁵ died with dyspnea and hemorrhagic pleuropneumonia within 20 to 48 hr post inoculation (PI). All pigs necropsied on 7 days in group 10⁵ and 10⁴ had focal fibrous pleuropneumonia. Histologically, pulmonary lesions were classified into three stages: purulent, acute and subacute. Fatal cases in group 10⁵ had purulent lesion composed of severe edema, hemorrhage and necrobiosis of alveoli, with mononuclear cells infiltration in the dilated interlobular. The fatal case in group 10⁴ had acute pulmonary lesion composed of focal or linear infiltration of round and fusiform cells that frequently showed swirling pattern in alveoli. The surviving cases in group 10⁵ and 10⁴ had subacute lesion composed of multifocal pulmonary necrosis surrounded by fibrous tissue. The swirling pattern was clearly seen in demarcation zone. Immunohistochemically, App antigens scattered as intact bacteria in alveoli, dilated interlobular septa and pleura, and lymph vessels in purulent and acute lesions. Areas of necrosis were also stained weakly. Although no antigen was detected in cytoplasm of macrophages and infiltrated cells in purulent lesions, App antigen was detected as positively stained mass in cytoplasm of some macrophages in acute lesions. In subacute lesions, App antigens were recognized as intact bacteria in necrotic areas and among the swirling pattern cells of demarcation zone. Macrophages had App antigens as a large mass of pigment in the cytoplasm in area of fibrosis. — KEY WORDS: Actinobacillus pleuropneumoniae, immunohistochemistry, pleuropneumonia, swine.


Actinobacillus pleuropneumoniae (App) is a causative agent of swine pleuropneumonia, in all parts of the world [19, 26]. In Japan, the first outbreak was reported in 1975 [20], and App serotype 2 was isolated from pneumatic lesions [3, 8, 9]. Recently App serotypes 1, 5, 6 and 8 were also isolated from pneumatic lungs [4, 7, 10, 14, 17]. It was reported that App serotype 1 had higher virulence than that of other serotypes [2, 23]. Although there have been many reports about the pathological feature on pneumatic lungs of pigs experimentally infected with App [1, 5, 11, 13, 16, 18, 27-29], a few reports described details of distribution of App antigen in the lesions [6, 15, 22]. The purpose of the present study was to determine the virulence of App serotype 1 and to evaluate the relationship between histologic lesions and the distribution of App antigen by use of immunoperoxidase procedure.

MATERIALS AND METHODS

Animals: Each group of 9-week-old pigs from specific pathogen free (SPF) sows were transferred to isolated housing units and fed on concentration feed without antibiotics and tap water ad libitum.

Bacterial strain: App serotype 1, HA-337 strain was originally isolated from lungs of an affected pig reared in Chiba Prefecture in 1989 [25], and supplied by Dr. T. Suzuki, Chiba Prefectural Institute of Animal Health, Japan. The organisms were cultured for 16 hr at 37°C under 10% CO2 atmosphere on heart infusion (HI) agar plus 2% chicken serum and 0.001% β-nicotinamide adenine dinucleotide (β-NAD). The organisms were then inoculated into HI broth with 5% chicken serum and 0.001% β-NAD. The cultures were incubated at 37°C for 6 hr with shaking, and centrifuged at 3,000 rpm for 30 min. The sediment was re-suspended in the original volume of phosphate-buffered saline (PBS), and diluted to each infection dose with PBS based on the standard curve by nephelometry and used for inoculum.

Experimental design: The nine animals were divided into 3 groups of pigs each, and they were inoculated with 1 ml of inoculum into both nostrils. The inoculums for each group contained 6.10 × 10⁶ (group 10⁶), 10⁵ (group 10⁵) and 10³ (group 10³) CFU/ml of the bacteria. They were evaluated for clinical signs and monitored daily for rectal temperature. Surviving animals were euthanized by electrocuted exsanguination 7 days post inoculation (PI).

Histopathology: Histopathology was performed on the liver, spleen, kidney, heart, lungs, trachea, tonsils, tracheobronchial, mediastinal, mandibular, and mesenteric lymph nodes. Tissues were fixed in 10% buffered neutral formalin and embedded in paraffin. The sections were cut at 4 μm, and were stained with hematoxylin and eosin.

Immunohistochemistry: The 4 μm-sections were placed on slide glasses coated with 0.05% poly-L-lysine, and were subjected to immunohistochemistry by avidin-biotin complex (ABC) procedure [21] using a commercially available kit ( Vectastain Elite ABC kit, Vector Laboratories, U.S.A.). Rabbit hyper-immune serum against Shope 4074, type strain of App serotype 1, was used for the primary antibody at a dilution of 1:125. For the negative control,
PBS was applied instead of primary antibody. The sections were counterstained with methyl green.

**Serology**: Blood was collected via the jugular vein before necropsy, and stored at −20°C. Serum antibody titers were examined by the enzyme-linked immuno-sorbent assay (ELISA) using monoclonal antibody specific to bacterial capsules of App serotype 1 [25].

**Bacteriology**: Tissues obtained at necropsy (Table 3) were inoculated onto brain heart infusion (BHI) isolation agar, and incubated at 37°C under 10% CO₂ atmosphere overnight. Isolates were identified by agglutination test using rabbit antiserum against Shope 4074.

**RESULTS**

**Clinical signs**: As shown in Table 1, none of pigs in group 10⁢³ showed clinical signs after challenge exposure, whereas 1 pig (No. 5) in group 10⁢⁰ and 2 pigs (Nos. 7 and 9) in group 10⁷ died within 48 hr PI. All of those inoculated with 10⁢³ and 10⁷ exhibited some depression, anorexia, pyrexia (40.0–42.0°C), and polypnea with dyspnea in 12 hr to 1 day PI. Dead cases showed severe respiratory distress, but others recovered on days 4 to 5 PI.

Before inoculation, all pigs in each group had low antibody titers (1:100–1:400) to App serotype 1 (Table 1). Surviving animals in groups 10⁷ and 10⁹ showed titers of 1:800–1:3,200 on day 7 PI, while no increase in antibody titers was seen in group 10⁷. Unfortunately, we could not examine the fatal cases for antibody titers.

**Gross findings**: Distribution patterns of pneumonic lesions are summarized in Fig. 1. Fatal cases No. 7 and No. 9 showed lobar hemorrhagic pleuropneumonia with a large amount of thoracic exudate, and a dead case No. 5 had hemorrhagic and necrotic pleuropneumonia with yellowish fibrinous exudation on the pleura and a large amount of dark reddish fluid in the thorax. Surviving cases in groups 10⁷ and 10⁹ had numerous dark reddish or gray reddish nodular lesions with fibrinous pleurisy. In the cut surface, irregular shaped coagulation necrosis were surrounded by fibrous capsule.

**Histopathology**: No lesions were detected in the lungs of pigs in group 10⁷. Pigs of Nos. 7 and 9 of group 10⁷ died within 25 hr PI had peracute pulmonary lesion, which consisted of severe hemorrhage, congestion and necrobiosis of alveoli. Edematous thickening and dilation of lymph vessels were seen in interlobular septa. Infiltration of degenerative mononuclear cells and neutrophils was observed in the border between alveoli and interlobular septa (Fig. 2). Some macrophages were infiltrated in the edematous area.

Pig No. 5 of group 10⁹, which died at 48 hr PI, had acute pulmonary lesion consisting of irregular, well-circumscribed regions of hemorrhagic consolidation or necrosis with exudation of fluid and fibrin in the alveoli and interstitium. Round or fusiform cells were seen within the alveoli and were making a zone that surrounded the bronchiole, vessels and necrotic lesions (Fig. 3). These cells were in slightly

<table>
<thead>
<tr>
<th>Inoculation dose</th>
<th>Case No.</th>
<th>Clinical sign</th>
<th>On Day 7 PI</th>
<th>Antibody titer*&lt;sup&gt;a&lt;/sup&gt; Pre exposure</th>
<th>Day 7 PI</th>
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<td>3</td>
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<td>alive</td>
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<td>dead in 48 hr</td>
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<td>800</td>
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<td>8</td>
<td>+</td>
<td>alive</td>
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<td>9</td>
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<td>dead in 20 hr</td>
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<sup>a</sup> −: no clinical signs, +: depression, anorexia and dyspnea.

<sup>b</sup> N.E.: not examined.

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![Fig. 1. Distribution of pneumonic lesions on the dorsal surface of the lungs.](image-url)
swirling pattern. A few neutrophils and macrophages were seen in the adjacent alveoli. Fibrino-edematous thickening of pleura and interlobular septa was also seen with a number of neutrophils and some macrophages.

The surviving pigs of group $10^4$ and $10^7$ had subacute lesion consisting of numerous area of coagulation necrosis surrounded by fibrous layer as well as darkly stained zones in an inner layer of fibrosis. The swirling pattern of the zones was more clear than that of acute lesion (Fig. 4), and sequestrated lesions were seen in lytic necrosis.

Severe epithelial desquamation was accompanied by the infiltration of neutrophils and mononuclear cells in the subepithelium in the trachea and bronchi of the dead cases. Lymphocytic depletion was seen in lymphatic follicles of the spleen, tonsil, tracheobronchial, and mesenteric lymph nodes. In the surviving cases of group $10^4$ and $10^7$, there were enlargement of follicles with severe neutrophil infiltration in perifollicular spaces of the tracheobronchial and mesenteric lymph nodes.

**Immunohistochemistry:** The distribution of App antigens is summarized in Table 2. In the peracute pneumatic lesions of pig No. 9, App antigens were recognized as intact bacteria in edematous fluid in dilated interlobular septa, pleura and in lymphatic vessels (Fig. 5). App organisms were also stained in the alveoli of the hemorrhagic and necrotic lesion (Fig. 6). In addition, the areas of necrosis were stained weakly. No antigen was detected in cytoplasm of macrophages and infiltrated cells of interlobulus. App antigens were also seen as intact bacteria on the surface of degenerated epithelium of trachea and in cell debris of bronchi. In the acute pneumatic lesion of pig No. 5, App antigens were recognized as intact bacteria in the alveolar edematous fluid and more frequently in the interlobular septa, pleura, lymphatic vessels. App antigen was detected as positively stained masses in cytoplasm of some macrophages. In subacute pneumatic lesion of pig No. 4, weak diffuse positive staining against App was detected in the parenchymal necrotic areas, and intact bacteria scattered.

Fig. 2. Interlobular septal thickening with severe edema (right), and zonal infiltration of mononuclear cells surrounding necrobiosis lobule in the peracute pneumatic lesion of pig No. 9 (group $10^7$). HE, × 56.

Fig. 3. Caudal lobe of the right lung of pig No. 5 (group $10^4$) at the acute stage. Round or fusiform cells are seemed to be in slightly swirling pattern (arrow). HE, × 560.

Fig. 4. The round or fusiform cells surrounding necrotic tissues in the subacute pneumatic lesion of pig No. 4 (group $10^3$) reveal a swirling pattern. HE, × 560.
in these areas. App antigens were located as intact bacteria in the darkly stained zones that surrounded the necrotic area (Fig. 7). Although App antigens were recognized among the swirling pattern cells, nearly unphagocytosed by these cells. In outer zones as well as fibrous layers and small lymph vessels, macrophages had App antigens as a large mass of pigment in the cytoplasm (Fig. 8). The antigen was also seen as intact bacteria in cell debris of bronchioli.

In Nos. 9 and 5, App antigens were recognized as intact bacteria in the mediastinal, intermediate and subcapsular sinus, cortex and follicles of the tracheobronchial lymph nodes. Some reticular cells had App antigens within their cytoplasm as small masses of pigment. App antigens were also detected as intact bacteria among cell debris, in epithelium of crypt, cortex and peripheral center of the tonsil. On the other hand, in No. 4, App antigens were remarkably stained as masses of pigment in medullary and intermediate sinus and follicles in the tracheobronchial lymph node (Fig. 9). App antigens were seen as intact bacteria among cell debris in fossula of the tonsil.

Bacteriology: As shown in Table 3, the surviving cases of groups 10^6 and 10^7, App was isolated from some of the recovering lobes in affected lungs, and tonsil of No. 4 of group 10^6. In the dead cases of groups 10^6 and 10^7, App was isolated from all lobes of lungs, pleural fluid, trachea, tracheobronchial lymph nodes and tonsil.

### DISCUSSION

In the present study, clinical diseases were observed in pigs intranasally inoculated with 10^5 and 10^7 CFU of App 1. This result was consistent with that of Shope [29] who produced a fatal disease in SPF pigs by intranasal inoculation of 10^4 and 10^5 CFU of App 1. It was confirmed that virulence of the bacteria used here was almost same as the one used by Shope [29]. The mortality rate in pigs inoculated with 10^5 CFU of App 1 was 66.6% in the present study, as it was in the SPF pigs [12, 23, 27]. The App isolates vary in virulence among each serotype [23]; mortality rate of other strain was 0% (10^5.5-6.1 CFU of serotypes 2 and 3) 2, 0 or 50% (2.5 x 10^6 CFU of serotype 2) and 0% (2.5 x 10^6 CFU of serotypes 3 and 7) [23]. Therefore, the virulence of the present strain was higher than that of these serotypes [2, 23].

Generally pneumonic changes in swine pleuropneumonia were classified into three types of lesion: peracute, acute and subacute lesions [13, 16, 18, 24]. A peracute lesion composed of lobar hemorrhagic pleuropneumonia with bacteremia was observed only in group 10^7. On the other hand, acute and subacute lesions were observed in the dead or surviving cases of groups 10^6 and 10^7. This histopathological typing might depend on the time after the inoculation [13, 16], and infection dose [23]. The surviving pigs in the subacute stage may have resulted from individual susceptibility and the number of bacteria reaching the terminal respiratory area. Finally, parenchymal necrotic lesions might be fully circumscribed by fibrous tissue and become sequester or abscess in chronic stage [18, 24]. Since live bacteria were isolated from lung lesions, the surviving pig may become a carrier and recur pleuropneumonia opportunistically.

In the peracute lesion, we recognized mononuclear cells and neutrophils. The origin of round or elongated cells appearing in early stage lesions was considered to be neutrophils [1, 11], and play a significant role in development of pulmonary lesions of App [11]. Further study is needed to evaluate the early stage lesions of App serotype 1, and the relation of mononuclear cells and neutrophils.

It has been described that App might spread from the pulmonary parenchyma to pleura through lymph vessels [1, 29], in which the existence of App antigen was proved [6, 15, 22]. In the dead cases of the present study, intact App were seen in the alveolar and interlobular fluid, as well as in the lymph vessels of interlobular septa and pleura in peracute and acute lesion. Therefore, we considered that App might be able to spread through not only lymph vessels [15] but also edematous fluid. Since the App were not phagocytosed by round or fusiform cells, these cells seemed to have dysfunction of phagocytosis probably due to effect of App toxins [26]. Parenchymal necrotic lesions were slightly stained by ABC procedure as in the previous study [22]. The finding suggested that the bacterial component including App toxin was diffusely existed in the necrotic area. App antigens were also recognized in parenchyma of tracheobronchial lymph nodes and tonsils. These findings indicated the invasion and proliferation of App bacteria in these tissues. In one surviving case No. 4, App antigens were detected in the necrotic lesions and darkly stained zone as in the previous study [15]. Since App were not phagocytosed by swirling pattern cells of the zone, it was suggested that these cells were degenerated or dysfunctioned.
Fig. 5. App antigens were recognized as intact bacteria in the dilated interlobular septa and lymph vessel in peracute pneumonic lesion of pig No. 9 (group 10⁷). ABC procedure. × 280.

Fig. 6. App antigens were also recognized as intact bacteria (arrows) in alveoli in the peracute pneumonic lesion of pig No. 9 (group 10⁷). Inflammatory fluid was also stained weakly. ABC procedure. × 560.

Fig. 7. App antigens were recognized as intact bacteria (arrows) in the swirling pattern cell zone in the subacute lesion of pig No. 4 (group 10⁷). ABC procedure. × 280.

Fig. 8. App antigens were also recognized as large masses of pigment in macrophages (arrows) in outer zone of swirling pattern cells of pig No. 4 (group 10⁷). ABC procedure. × 560.

Fig. 9. App antigens were mainly recognized in the follicle of the tracheobronchial lymph node of pig No. 4 (group 10⁷). ABC procedure. × 140.
Table 3. Bacteriologic findings

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<th>Fig</th>
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a) D: Dead, b) Not examined, c) Others: Liver, Spleen, Kidneys, Mandibular Lm., Mesenteric Lm. d) --: No growth, +: Growth of organisms compatible with App.

of phagocytosis as well as round or fusiform cells. On the other hand, App were phagocytosed by macrophages in outer fibrotic lesions; it was suggested that phagocytosed App flowed into follicles of tracheobronchial lymph node through lymph vessels. Immunological response may occur in this region [22].

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