Detection of Antibodies against *Actinobacillus pleuropneumoniae* Serotypes 1, 2, 5 and 7 Using the Immunohistochemical Staining

Hisayuki TAKASHIMA, Hiroki SAKAI, Tokuma YANAI and Toshiaki MASEGI

Department of Veterinary Pathology, Faculty of Agriculture, Gifu University, 1-1 Yanagido, Gifu 501-1112, Japan

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ABSTRACT. Whole cells of *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) serotype 1, 2, 5 or 7 attached to fibrins were fixed in 10% neutral buffered formalin and embedded in paraffin. The sections on a slide glass were stained by the avidin-biotin complex immunoperoxidase (ABC) method. Test sera were applied to sections as primary antibodies. The serum antibodies against *A. pleuropneumoniae* (serotypes 1, 2, 5 and 7) were measured by the ABC method and complement fixation (CF) test. There was good correlation between the ABC and CF tests. The present results indicate that the immunohistochemical staining is as useful as the CF test for the detection and quantification of antibody in swine sera.—**KEY WORDS:** *Actinobacillus pleuropneumoniae*, antibody, immunohistochemistry.

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Pleuropneumonia in pigs caused by *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) continues to be a serious threat to the pig industry throughout the world. The acute and subacute states of the disease are characterized by fibrinohemorrhagic pleuropneumonia and coagulative necrosis [9–12]. This disease occurs in pigs of all ages, and the associated mortality is high on the farm where the pigs are exposed to the primary infection. Naturally infected pigs with *A. pleuropneumoniae* have pulmonary lesions and may become subclinical carriers of the pathogen [10].

Antibodies against *A. pleuropneumoniae* were demonstrated by the immuno diffusion test [2], complement-fixation (CF) test [3], enzyme-linked immunosorbent assay (ELISA) [1, 5–7] and inhibition enzyme immunoassay (EIA) [13, 14]. The CF test has been favored as the routine method for serodiagnosis of *A. pleuropneumoniae* infections [3]. However, the CF test is a complicated, time-consuming and low sensitivity method.

In the present study, we demonstrated the specific antibodies against *A. pleuropneumoniae* in the experimentally and naturally infected pigs by using the avidin - biotin complex immunoperoxidase (ABC) method, and its specificity was compared to that of CF test.

A. pleuropneumoniae (serotypes 1, 2, 5 and 7) and sera of specific-pathogen-free (SPF) pigs experimentally infected with *A. pleuropneumoniae* (serotypes 1, 2, 5 and 7) were obtained by courtesies of Dr. T. Morozumi (National Institute of Animal Health, Japan) and Dr. E. Oishi (Division of Veterinary Microbiology, Kyoto Biken Laboratories, Japan). One strain of *Actinobacillus suis* (*A. suis*) and one of *Haemophilus parasuis* (*H. parasuis*) were also used in the studies as antigens. The one hundred twenty sera examined were collected from 6 different swine farms with or without a clinical history of *A. pleuropneumoniae* infection. The serum antibodies of these samples against *A. pleuropneumoniae* serotypes 1, 2, 5 and 7 were measured by the ABC and CF tests. Both tests were performed as follows: *Immunoperoxidase staining (enzyme antibody method)*: The calf blood was dispensed to tubes, and

centrifuged at 3,000 rpm for 5 min at 4°C. Whole cells (10^8 cfu/ml $\times$ 0.5 ml) of four serotypes of *A. pleuropneumoniae*, *A. suis* or *H. parasuis*, which were used as antigens for the ABC method, were added to plasma (6 ml) in the tube at 4°C. After incubating the tubes for 60 min at 37°C, fibrins were separated from supernatant fluid. Whole cells of four serotypes of *A. pleuropneumoniae*, *A. suis* or *H. parasuis* attached to fibrins were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut at 3–4 μ m. Three or four sections were put on each slide glass. Bacterial antigens were demonstrated by the ABC method, using a Vectastain ABC kit (Vector Lab., U.S.A.) [4]. Briefly, sections on slides were deparaffinized, rehydrated through graded series of ethanol, quenched with 0.3% H₂O₂ in absolute methanol to block endogenous peroxidase for 30 min at room temperature, and rinsed with phosphate - buffered saline (PBS) (pH 7.5) three times for 5 min each at room temperature. After washing, sections on slides were treated with 0.1% trypsin in PBS for 10 min at 37°C, and then rinsed with PBS three times for 5 min each at room temperature. Sections on slides were treated with 2% normal goat serum in PBS for 20 min at room temperature. The test sera of pigs infected experimentally or naturally with *A. pleuropneumoniae* were tested at serial twofold dilutions ranging from 1:25 to 1:25,600 in a plastic microplate. After removing the normal goat serum, sections on slides were incubated with 0.1 ml of each dilution of the test sera as primary antibody for 30 min at room temperature, and then rinsed with PBS three times for 5 min each at room temperature. A dilution of biotinylated goat anti-swine IgG (Vector Lab., U.S.A.) was applied as secondary antibody for 30 min at room temperature, and the slides were rinsed with PBS as described above. Thereafter, the slides were incubated with avidin-biotin complex for 30 min at room temperature, and peroxidase activity was demonstrated by treating them with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Japan). After mounting, the sections were examined under the light microscope. The ABC titers were recorded as the highest

Fig. 1a. Immunohistochemical staining for whole cells of *A. pleuropneumoniae* serotype 1 attached to fibrins. Many fibrins with whole cells strongly reacted with *A. pleuropneumoniae* serotype 1 antibody. Dilution of the positive reference serum is 1:500. Immunoperoxidase staining, $\times 700$.

Fig. 1b. Immunohistochemical staining for whole cells of *A. pleuropneumoniae* serotype 1 attached to fibrins. Dilution of the negative reference serum is 1:500. Immunoperoxidase staining, $\times 700$.

serum dilutions with specific staining. Positive and specific staining in the fibrins with positive serum strongly reacted as compared with negative reference serum.

CF test: The CF test was performed as described by Gunnarsson [3]. Microtitration plates with U-shaped bottoms were used in all tests. Guinea pig serum was used as complement in $1.5 \times$ concentration, which gave 50% hemolysis of the sheep red blood cells (RBC). Sera from calves were added to the guinea pig complement. Sheep RBC were used in a 2% concentration in Alsever's solution. The test sera from swine were inactivated in a water bath at 62°C for 20 min. Hemolysin rabbit antiserum prepared against sheep RBC was used. To each well, 0.025 ml of PBS was added. Two-step dilutions were performed by dropping 0.025 ml of test sera of pigs infected experimentally or naturally with *A. pleuropneumoniae* into the first wells. Each well received 0.025 ml of antigen. A total of complement was added to all well. Plates were incubated at 4°C overnight. A total of 0.05 ml of hemolysin mixed with equal amounts of 2% sheep RBC was dropped into each well. Plates were incubated at 37°C for 60 min before reading. Reactions were read with the eye in a convex mirror. The CF titers were recorded as the highest serum dilutions showing $\geq 50\%$ fixation.

Antibodies against *A. pleuropneumoniae* serotypes 1, 2, 5 and 7 were detected by the ABC method. Whole cells of each serotype of *A. pleuropneumoniae* attached to fibrins

strongly reacted with corresponding antibody against *A. pleuropneumoniae* (Fig. 1a, 1b). The serum antibodies against *A. pleuropneumoniae* (serotypes 1, 2, 5 and 7) were measured by the ABC and CF tests. A good correlation was found between the ABC and the CF tests, but titers were higher in the ABC test than in the CF test for the weakly positive sera (Fig. 2a, 2b). Because ABC titers were about 300 times higher than CF titers, positive titers were $\geq 1:4$ for the CF test, and $\geq 1:1,200$ for the ABC method. Sera of SPF pigs experimentally infected with *A. pleuropneumoniae* (serotypes 1, 2, 5 and 7), one strain each of *A. suis*, and *H. parasuis* were examined by the ABC method using different antigens of *A. pleuropneumoniae*, *A. suis* and *H. parasuis*. Cross-reactions were observed among serotypes of *A. pleuropneumoniae*, and the degree of cross-reactivity varied somewhat among different batches of the antigens. Whereas, no cross-reactions were present between *A. suis* or *H. parasuis* and swine antisera to four serotypes of *A. pleuropneumoniae* (Table 1). Results of the ABC method on 120 serum samples from 6 different farms were described. In case of *A. pleuropneumoniae* serotype 1, of 80 sera from 4 swine farms with a clinical history of *A. pleuropneumoniae* infection, 11 (13.8%) sera had ABC titers of $\geq 1:1,200$. In case of *A. pleuropneumoniae* serotype 2, of 80 sera from 4 swine farms with a clinical history of *A. pleuropneumoniae* infection, 45 (56.3%) sera had CF titers of $\geq 1:4$, while 48 (60%) sera had ABC titers of $\geq$

Fig. 2a. Comparison of titers between the ABC and CF tests, in case of *A. pleuropneumoniae* serotype 1. The serum antibodies against *A. pleuropneumoniae* serotype 1 were measured by the ABC and CF tests. The 30 serum samples collected from pigs experimentally and naturally infected with *A. pleuropneumoniae* serotype 1 were titrated and analyzed by the two tests. The ABC titers plotted against corresponding the CF titers. Good correlation was found between them. The regression line ($r=0.81$) was calculated as follows: y (ABC titer) = $293 \times (\text{CF titer}) + 204$. Antigen is *A. pleuropneumoniae* serotype 1.

Fig. 2b. Comparison of titers between the ABC and CF tests, in case of *A. pleuropneumoniae* serotype 2. The 30 serum samples from pigs were titrated and analyzed by the two tests. There was good correlation between them. The regression line ($r=0.74$) was calculated as follows: y (ABC titer) = $282 \times (\text{CF titer}) + 197$. Antigen is *A. pleuropneumoniae* serotype 2. Comparisons of titers in *A. pleuropneumoniae* serotype 5 ($r=0.74$, $y=267x + 181$) and serotype 7 ($r=0.78$, $y=255x + 168$) were similar to *A. pleuropneumoniae* serotype 2.

1:1,200 (Table 2a, 2b). With field sera from herds infected with *A. pleuropneumoniae*, the ABC method appeared to be more sensitive than the CF test.

The ABC method described here seems to be as useful as the CF test for the detection and quantification of *A. pleuropneumoniae* antibody in swine sera. Unlike the CF test, however, this method is simple and can be performed in 4 hr, similarly to the ELISA [1, 5–7] and EIA [13, 14]. In addition, the method is highly specific and sensitive, and low levels of antibodies can be detected more frequently by this method than by the CF test. Hence it appeared that the ABC method could be used in a herd health monitoring program for detecting of *A. pleuropneumoniae* infections. Furthermore, formalin-fixed specimens on slide glasses can be preserved for a long time. The only drawback in this method is inability to deal with a great many samples at a time.

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Table 1. Cross-reactions in the ABC test by different serotype antigens

Antiserum	Antigen					
	<i>A. pleuropneumoniae</i> serotype				<i>A. suis</i>	<i>H. parasuis</i>
	1	2	5	7		
<i>A. pleuropneumoniae</i>						
Serotype 1	12,800	200	—	—	—	—
Serotype 2	—	12,800	200	—	—	—
Serotype 5	—	200	12,800	—	—	—
Serotype 7	—	—	—	6,400	—	—
<i>A. suis</i>	—	—	—	—	3,200	—
<i>H. parasuis</i>	—	—	—	—	—	6,400

Data are expressed as reciprocal of highest serum dilutions showing specific staining; — = no reaction.

Table 2a. Result of the ABC test for swine sera from six herds tested with different serotype antigens

Antiserum	Antigen			
	<i>A. pleuropneumoniae</i> serotype			
	1	2	5	7
Herds with seropositive swine ^{a)}				
A	0/20	12/20	0/20	0/20
B	5/20	14/20	2/20	0/20
C	4/20	14/20	3/20	0/20
D	2/20	8/20	0/20	0/20
Herds with seronegative swine ^{b)}				
E	0/20	0/20	0/20	0/20
F	0/20	0/20	0/20	0/20

Data are expressed as No. of positive reactors/No. of swine tested in herd.

a) Four herds (designated A to D) had serious clinical problems with infections due to *A. pleuropneumoniae*.

b) Two herds (E and F) were clinically healthy and had no history of *A. pleuropneumoniae* infection.

c) Positive: ABC titer of $\geq 1:1,200$.

Table 2b. Result of the CF test for swine sera from six herds tested with different serotype antigens

Antiserum	Antigen			
	<i>A. pleuropneumoniae</i> serotype			
	1	2	5	7
Herds with seropositive swine ^{a)}				
A	0/20	11/20	0/20	0/20
B	4/20	14/20	1/20	0/20
C	4/20	13/20	3/20	0/20
D	1/20	7/20	0/20	0/20
Herds with seronegative swine ^{b)}				
E	0/20	0/20	0/20	0/20
F	0/20	0/20	0/20	0/20

Data are expressed as No. of positive reactors/No. of swine tested in herd.

a) Four herds (designated A to D) had serious clinical problems with infections due to *A. pleuropneumoniae*.

b) Two herds (E and F) were clinically healthy and had no history of *A. pleuropneumoniae* infection.

c) Positive: CF titer of $\geq 1:4$.