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Application of an enzyme-linked immunosorbent assay for detection of antibodies to *Actinobacillus pleuropneumoniae* serovar 15 in pig sera

Kaho TESHIMA[1], Jina LEE[1], Ho TO[1]*, Takashi KAMADA[1], Akihiro TAZUMI[1], Haruna HIRANO[2], Minoru MARUYAMA[3], Torata OGAWA[4], Shinya NAGAI[1], Conny TURNI[5] and Nobuyuki TSUTSUMI[1]

[1]Nippon Institute for Biological Science, 9-2221-1 Shinmachi, Ome, Tokyo 198-0024, Japan
[2]Yamanashi Prefecture Eastern Livestock Hygiene Service Center, 1001-1 Karakashiwa, Ishiwa, Fuefuki, Yamanashi 406-0034, Japan
[3]Yamanashi Prefecture Livestock Dairy Technology Center, 963-1 Otokuro, Chuou, Yamanashi 409-3812, Japan
[4]Fukuoka Prefecture Central Livestock Hygiene Service Center, 4-14-5 Hakozakihutou, Higashi, Fukuoka, Fukuoka 812-0051, Japan
[5]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Ecosciences Precinct, Dutton Park, QLD, Australia

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ABSTRACT. An indirect enzyme-linked immunosorbent assay (ELISA) using lipopolysaccharide extract as antigen was evaluated for detection of antibodies to *Actinobacillus pleuropneumoniae* serovar 15. The serovar 15 ELISA had a higher sensitivity and specificity than latex agglutination test for 63 and 80 sera from pigs experimentally infected and not infected with *A. pleuropneumoniae*, respectively. When the serovar 15 ELISA was applied to 454 field sera, high rates of seropositivity were found in pigs from farms infected with *A. pleuropneumoniae* serovar 15, but not in those from farms free of *A. pleuropneumoniae* serovar 15. The results suggest that the serovar 15 ELISA may be useful for the serological surveillance of infection with *A. pleuropneumoniae* serovar 15.

KEY WORDS: *Actinobacillus pleuropneumoniae*, ELISA, LPS, pig, serovar 15

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, an economically important bacterial infection of swine. To date, serovars 1 to 15 have been recognized mainly on the basis of the antigenic, structural and genetic characteristics of capsular polysaccharides (CPS) and the O-polysaccharide (O-PS) [3, 5, 9, 10, 18–20, 25], and serovar 16 was proposed based on antigenic and genetic characteristics [1, 20]. Moreover, the virulence associated with certain serovars varies between countries [3, 5].

It has been shown that serovar-specific serological tests are an important tool for understanding the epidemiology of an outbreak, for monitoring the occurrence of different serovars in infected herds and for detection of subclinical infected pigs. The complement fixation, tube agglutination, LA and ELISA tests have been used for detection of antibodies against *A. pleuropneumoniae* [2, 3, 5–7, 12, 14, 17]. Among these serodiagnostic tests, ELISA has been considered to be easier to use, automated and the most sensitive and specific. Recently, ELISAs based on crude boiled extract (CBE), CPS or lipopolysaccharide (LPS) antigens have been developed for detection of antibodies against *A. pleuropneumoniae* [2, 3, 5, 7, 12, 17].

In Japan, serovar 2 is the most predominant, followed by serovars 1, 5 and 15. Disease due to *A. pleuropneumoniae* serovar 15 has been found in Aichi (unpublished data), Chiba, Ehime, Fukuoka, Hokkaido and Kagawa prefectures of Japan [8, 13, 16, 21, 24]. Thus, the development of more effective vaccines against the infection by serovar 15, and of rapid and specific serological tests for detection of antibodies to serovar 15 are highly desired. Currently, a commercial ELISA specific to serovars 3-6-8-15 offered by Biovet (Saint-Hyacinthe, Québec, Canada) under the name Swinecheck®mix-APP ELISA is available in some countries [17] but not in Japan. In the present study, we evaluated an ELISA based on the LPS of serovar 15 for detection of antibodies to *A. pleuropneumoniae*. The serovar 15 ELISA were evaluated and compared to the latex agglutination (LA) test for sensitivity and specificity by using sera from experimentally infected and non-infected pigs. Then the test was applied

*Correspondence to: To, H.: toho@nibs.or.jp

Table 1. Sensitivity, specificity, and predictive value of ELISA and LA for 143 sera

	ELISA result		LA result	
	Positive	Negative	Positive	Negative
Sera from pigs experimentally infected with *A. pleuropneumoniae* serovars 3, 6, 8 and 15	29	3	24	8
Sera from pigs not infected with *A. pleuropneumoniae* serovars 3, 6, 8 and 15	0	111	0	111
Sensitivity (%)	91		75	
Specificity (%)	100		100	
Positive predictive value (%)	100		100	
Negative predictive value (%)	97		93	

to the field sera for detection of antibodies to *A. pleuropneumoniae* serovar 15 from infected pigs.

LPS was extracted from *A. pleuropneumoniae* serovar 15 reference strain HS143 using the lysozyme-phenol-water method as previously described [11, 24]. Briefly, cell pellet was suspended in 50 mM sodium phosphate buffer, pH 7.0 containing 5 mM EDTA and 0.05% sodium azide, treated with lysozyme and then stirred overnight at 4°C. The suspension was treated sequentially with ribonuclease, deoxyribonuclease, proteinase K, and then extracted with 50% aqueous phenol at 68°C for 30 min. The solution was chilled on ice for 30 min and centrifuged. The upper aqueous phase was collected, extracted twice more and then the final aqueous phase was dialyzed against distill water at 4°C for 72 hr to remove residual phenol. The obtained LPS preparation was used as antigen in the serovar 15 ELISA and LA. The purity and antigenicity of the LPS preparation were confirmed by SDS-PAGE and immunoblotting, respectively [23].

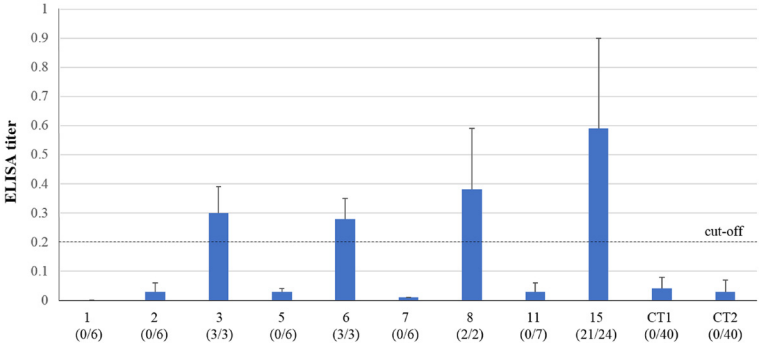

Fig. 1. Analysis of 63 and 80 sera from pigs experimentally infected and non-infected with *A. pleuropneumoniae*, respectively. Analysis was performed using an indirect ELISA with LPS from serovar 15 as antigen. Each bar represents ELISA titers (mean and SDs) of sera from pigs experimentally inoculated with particular serovar. The two last bars to the right represent pre-inoculation sera (n=40; CT1, control 1) and field sera collected from farms with no history of infection with serovars 3, 6, 8 and 15 (n=40; CT2, control 2) as negative controls, respectively. The dashed line indicates the cut-off value. Numbers in brackets indicate the number of positive sera by the serovar 15 ELISA per the number of sera tested for particular serovar.

The 0.9-μm polystyrene beads (Aica Kogyo Co., Ltd., Tanba, Japan) were used as carrier for LPS antigen. The coating of latex particle with the antigens and the LA test were performed as described previously [14].

The ELISA procedures were similar to those described previously [7, 12]. Briefly, the appropriate dilution of the LPS antigen was determined by checkerboard titration in microtiter plates (PolySorp, Thermo Scientific Nunc, Roskilde, Denmark) using carbonate buffer (0.5 M, pH 9.6). The plates were coated with the LPS antigen (50 μl/well) and incubated overnight at 4°C. The plates were washed with PBS-0.05% Tween20 (PBST) and blocked with PBST-1% BSA (200 μl/well) for 1 hr at 25°C. After washing once with PBST (200 μl/well), sera (1:100) diluted by PBST-1% BSA were added, and the plates were incubated for 30 min at 37°C. After washing twice with PBST, peroxidase-conjugated rabbit anti-swine IgG (H+L) (1:10,000; Rockland Immunochemicals Inc., Limerick, PA, U.S.A.) was added and allowed to react for 30 min at 37°C. The plates were washed twice with PBST and a chromogenic solution was allowed to react for 30 min at 30°C. The reaction was stopped with 4.0 N H_2SO_4. The optical density (OD) at 490 nm was measured with 650 nm as the reference. The ELISA titer was shown by the absorbance of sample serum to absorbance of positive reference serum (S/P) ratio, which was calculated as follows: (sample absorbance-negative reference absorbance)/(positive reference absorbance−negative reference absorbance). The absorbances obtained with the negative reference serum sample (collected from nonimmunized control pigs) were 0.00 to 0.07, and the absorbances obtained with the positive reference serum sample (collected from pigs experimentally infected with strain HS143) were approximately 1.0 under the respective conditions for the positive reference samples.

The 143 sera selected from our collection (Table 1) were used to evaluate the serovar 15 ELISA and to compare the ELISA and LA for detection of antibodies against serovar 15 as well as serovars 3, 6 and 8 which share common LPS O-polysaccharide antigen with seovar 15. Thirty-two and 31 sera (Fig. 1) from non-immunized control SPF pigs of vaccine trials inoculated intratracheally with serovars 3, 6, 8, 15 and 1, 2, 5, 7, 11, respectively, were collected at day 7 when the trials were terminated. In addition, 40 pre-inoculation sera from SPF pigs and 40 pig sera from farms with no history of infection with *A. pleuropneumoniae* serovars 3, 6, 8 and 15 were used as negative serum samples. The 40 pig sera from farms were collected from pigs immunized with commercial vaccines containing inactivated antigens of *A. pleuropneumoniae* serovars 1, 2 and 5; *Bordetella bronchiseptica (B. bronchiseptica)*; *Pasteurella multocida* toxin (PMT); *Erysipelothrix rhusiopathiae* (*E. rhusiopathiae*); *Haemophillus parasuis* (*H. parasuis*) serovars 2 and 5; *Mycoplasma hyopneumoniae (M. hyopneumoniae)*; porcine reproductive and respiratory syndrome

virus (PRRS) or porcine circovirus (PCV). The cut-off value was defined as the mean ELISA titer of 111 serum samples from non-infected pigs plus three times the standard deviation (SD). The sensitivity, specificity and predictive values of the ELISA were estimated, and compared to those of the LA. The specificity of the ELISA was also examined using pig sera against *H. parasuis* serovar 2, *E. rhusiopathiae* serovar 1a and *M. hyopneumoniae* (3 pig sera each).

A total of 454 field pig sera were collected from 14 farms, including growing pigs (4–6 months of age) and sows from 2 farms (A and B) naturally infected with serovar 15 and growing pigs (4–6 months of age) from 12 farms with no history of infection with *A. pleuropneumoniae* serovars 3, 6, 8 and 15.

The mean value of the ELISA titer plus 3 times the SD of 111 serum samples from animals with no history of infection with *A. pleuropneumoniae* serovars 3, 6, 8 and 15 was 0.14 but two and one out of these 111 sera had the ELISA values of 0.15 and 0.17, respectively. To yield high specificity, a cut-off value of 0.2 was chosen. At the cut-off value of 0.2; 29 out of 32 sera from pigs infected with serovars 3, 6, 8 and 15 were positive (Table 1). Whereas, at the LA titer of ≥8; 24 out of 32 sera were positive. Of eight LA-negative serum samples, five were positive by the ELISA. Good correlation (an R-squared value of approximately 0.94) was found between the ELISA and LA tests, however, the seropositivity rate was higher in the ELISA test than in the LA test for the weakly positive sera (Fig. 2).

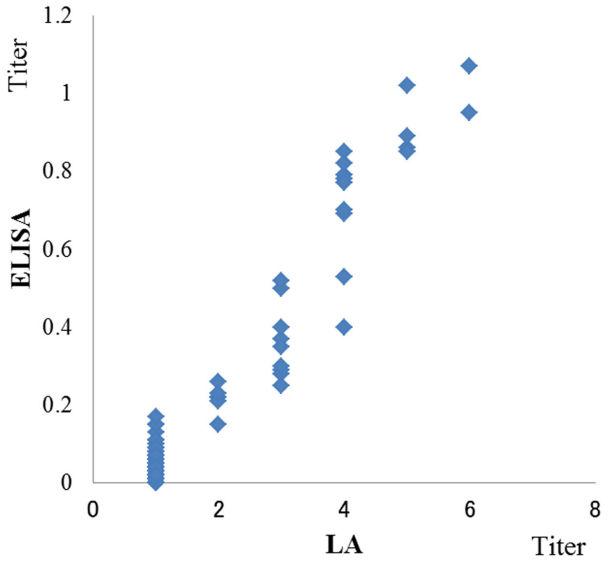

Fig. 2. Comparison of titers between the ELISA and LA tests. The serum antibodies against *A. pleuropneumoniae* serovars 3, 6, 8 and 15 were measured by the ELISA and LA tests. The 143 serum samples (including the 80 negative control sera, 31 sera from pigs infected with serovars 1, 2, 5, 7 and 11; and 32 sera from pigs infected with serovars 3, 6, 8 and 15) were examined by the two tests. The ELISA titers of 143 sera were plotted against the corresponding LA titers. Good correlation was found between two tests. The regression line (an R-squared value=0.94) was calculated as follows: y (ELISA titer)=0.2081 ×log2 (LA titer) + 0.1812. The LPS antigen of *A. pleuropneumoniae* serovar 15 was extracted by using the lysozyme-phenol-water method.

Specificity, sensitivity and predictive values of the ELISA using the 63 and 40 sera from SPF pigs experimentally infected and prior to infection with *A. pleuropneumoniae*, respectively, and 40 pigs from farms without a history of *A. pleuropneumoniae* infection are summarized in Table 1. Sera from animals infected with *H. parasuis*, *E. rhusiopathiae, M. hyopneumoniae* and those from pigs vaccinated with commercial vaccines containing inactivated antigens of *A. pleuropneumoniae* serovars 1, 2 and 5; *B. bronchiseptica*; PMT; *E. rhusiopathiae*; *H. parasuis* serovars 2 and 5; *M. hyopneumoniae*; PRRS or PCV had no reaction in the serovar 15 ELISA (mean ELISA titers ranged from 0.02 to 0.04).

The results of the ELISA for the detection of antibodies to serovar 15 in 454 field sera are shown in Table 2. In the affected farms (disease due to serovar 15 was confirmed by bacterial isolation), 50.9 (farm A) and 88.7% (farm B) of sera tested were positive, but none of 215 sera from the unaffected farms had detectable antibodies to *A. pleuropneumoniae* serovar 15. Out of positive sera, 34 (47.2%) of 72 and 58 (82.9%) of 70 sera were collected from farms A and B in 9- and 5-year periods, respectively, before the occurrence of the disease.

The CBE, CPS, and long chain LPS (LC-LPS) antigens prepared from different serovars have been used for detection of antibodies to the corresponding serovars [2, 3, 5]. The CPS antigen was found to be highly specific but production of CPS was time-consuming, expensive and cumbersome [3, 5]. The LC-LPS antigens purified by various methods were proved to have better specificity and sensitivity than CBE antigen [3]. Observation that the LC-LPS and LPS short O-chains of serovar 15 reacted with sera from pigs experimentally infected with serovars 3, 6, 8 and 15 but not with those from pigs infected with serovars 1, 2, 5, 7 and 11 is consistent with data from reports by Perry *et al*. [18, 19]. Those investigators showed that the observed partial serological cross-reactions between serovars 3, 6, 8 and 15 probably arise from antibodies to LPS because the chemical structures of the LPS O-chain of serovars 3, 8 and 15 were identical but slightly different from that of serovar 6 [18, 19]. Previously, an indirect MIX-ELISA using the LC-LPS of serovars 2, 6 and 12 as antigen reportedly detected antibodies to *A. pleuropneumoniae* serovar 8 [7]. The observations on the chemical structures of the LPS O-chains of serovars 3, 8, 15 and 6 [18, 19] support the idea that the serovar 15 LPS antigen, containing mainly the LC-LPS and short O-chain, is more suitable than serovar 6 LPS antigen for detection of antibodies to serovar 15. Additionally, the antigen could be extracted and obtained in large quantities in a few steps.

In our study, the ELISA test was evaluated by comparison with the LA test, which is shown to be highly specific and more sensitive than other serological tests (tube agglutination, agar-gel immunodiffusion and complement fixation tests) [14]. The results confirmed that the ELISA and LA using the LPS antigen obtained from serovar 15 detected not only the antibodies to serovar 15 but also those to serovars 3, 6 and 8 in pig sera. There were no cross-reactions with sera from pigs infected with serovars 1, 2, 5, 7 and 11. Our study with the ELISA test, though still limited, also confirmed this specificity. In the current study, seroconversion was detected at seven days post-inoculation and the positive percentages varied from 75% (24 of 32) and 91% (29 of 32) of the animals inoculated with serovars 3, 6, 8 and 15 by the LA and ELISA, respectively. A good correlation was found between the two

Table 2. The positive incidence of *Actinobacillus pleuropneumoniae* serovar 15 antibodies using the ELISA test in field pig farms

Farms (Number of farms)	The time of sample collection	Number of pigs tested (Sows + Growing pigs[a])	Number of positive pigs (Sows + Growing pigs)	Positive (%)	Mean ELISA titer in pigs tested	SD[b]
Affected (2)						
A	During the occurrence of the disease (2013)	34 (16+18)	20 (8+12)	58.8	0.39	0.31
	Before the occurrence of the disease (2004–2012)	72 (48+24)	34 (24+10)	47.2	0.26	0.19
	Total	106 (64+42)	54 (32+22)	50.9	0.36	0.28
B	During the occurrence of the disease (6/2015–9/2015)	63 (23+40)	60 (22+38)	95.2	0.64	0.41
	Before the occurrence of the disease (2010–5/2015)	70 (9+61)	58 (9+49)	82.9	0.66	0.35
	Total	133 (32+101)	118 (31+87)	88.7	0.66	0.36
Free (12)		215	0	0	0.03	0.03

a) Growing pigs in Farm A and Farm B were 4–5 and 6 months of age, respectively. b) SD, standard deviation.

tests, but five serum samples gave discordant results. A negative LA test result with positive ELISA was found for the 5 samples from animals inoculated with serovars 3 (n=1), 6 (n=1), 8 (n=1) and 15 (n=2). These negative results may be explained by the failure of the LA test to detect antibodies in serum samples probably taken too early in the course of the disease. In addition, three samples from the animals inoculated with low doses of serovar 15 were negative by both the ELISA and LA, respectively. No clinical symptoms and lung lesions typical for porcine pleuropneumonia were observed in these three animals. It seems that the animals in the present study remained uninfected perhaps due to a lower dose than required to induce infection, explaining the lack of detection of antibodies. These findings together with data reported by other investigators [2, 17] support the idea that virulence, inoculum dose and the heterogeneity in cellular and humoral immune responses among individuals contribute to clinical infection and seroconversion.

The results demonstrated here also indicate that the serovar 15 ELISA can be used advantageously for detecting antibodies to serovars 3, 6, 8 and 15 in infected animals and/or for evaluation of the potency of vaccines containing *A. pleuropneumoniae* serovar 15 as a component. So far, there is no serological test capable of differentiating animals infected with serovars 3, 6, 8 or 15. Therefore, for the serological survey positive reactions in the ELISA should be complemented by isolation and serotyping of the bacteria to determine which serovar caused the infection. Fortunately, *A. pleuropneumoniae* serovars 3, 6 and 8 have been rarely isolated from naturally infected pigs in Japan, whereas porcine pleuropneumonia due to serovar 15 has been found in various regions of Japan from 2003 [8, 13, 16, 21].

When the ELISA was applied to the field sera collected from farms affected by serovar 15 and those from farms with no history of infection with *A. pleuropneumoniae* serovars 3, 6, 8 and 15, high percentages of positive antibody were observed in animals from the affected farms, but not in animals from unaffected farms. In addition, in retrospective analyses of sera collected years before the occurrence of the disease, high percentages of samples with positive antibody were observed since year 2004. These findings together with data reported by other investigators [16, 21] support the idea that the infection due to serovar 15 has existed in the pig populations in Japan long before serovar 15 was identified.

Cross-reaction by the serovar 15 LPS antigen with sera from pigs infected with *A.suis* was not defined in the present study. Nevertheless, data from recent studies [4, 15, 17, 22] showed that except for similarities in the antigenic epitopes and structure of the LPS core-lipid A, the antigenic epitopes and structures of CPS and LPS of *A. suis* were different from those of *A. pleuropneumoniae*. Those studies provided suggestive evidence that the antibodies to *A. suis* reacted with the LPS core-lipid A of serovar 15 but not with CPS and other LPS antigens of serovar 15. A recent study from our laboratory [24] shows that the low molecular weight region, corresponding to the LPS core-lipid A, was not observed in the LPS preparation obtained by the lysozyme-phenol-water method. The antigen containing mainly O-chain LPS of serovar 15 seems to be, in our hand, the antigen that retains the best specificity while keeping a good sensitivity for detection of antibodies to serovar 15 by the ELISA.

In conclusion, the indirect ELISA based on the serovar 15 LPS antigen for detection of antibodies to serovar 15 in pig sera was evaluated. The results presented here suggested that this assay has an optimal sensitivity and a high specificity, which may be a useful tool for the serological surveillance for infection with serovar 15.

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