

## Serotyping reanalysis of unserotypable *Actinobacillus pleuropneumoniae* isolates by agar gel diffusion test

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**ABSTRACT.** We observed increasing unserotypable (UT) *Actinobacillus pleuropneumoniae* isolates using agar gel diffusion (AGD) test. To reanalyze their serovar, we performed rapid slide agglutination (RSA) test and multiplex PCR for 47 UT isolates. Of these, 25 were serovar 1 (UT-serovar 1), 20 were serovar 2 (UT-serovar 2) and 2 were serovar 15 (UT-serovar 15). We examined serotyping antigen extraction temperature to determine heat influence. UT-serovar 1 and 15 were influenced by heat, because their precipitation lines were observed in the case of low antigen extraction temperature. To investigate the relationship between antigenicity and genotype, we performed pulsed-field gel electrophoresis (PFGE) analysis using UT-serovar 2 and 15. The predominant PFGE pattern of UT-serovar 2 was identical to that of serovar 2.

**KEY WORDS:** *Actinobacillus pleuropneumoniae*, serotyping

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*Actinobacillus pleuropneumoniae* causes porcine pleuropneumoniae, which leads to significant economic losses in the pig industry worldwide [15]. Presently, *A. pleuropneumoniae* is divided into two biotypes based on the requirement for nicotinamide adenine dinucleotide and further subdivided into 15 serovars based on capsular antigens [1]. The prevalence of different serovars varies between countries [4, 5, 9–11, 15]. In 1980s, most field isolates in Japan were serovar 2 [6, 7, 16]. Subsequently, the isolation rate of various serovars, particularly 1 and 5, has increased substantially [13]. There has also been an increase in isolates that are unserotypable (UT) [13, 16], because no precipitation lines were observed between the antigen and antiserum in agar gel diffusion (AGD) test using autoclaved antigens (121°C for 1 hr) [16]. In this study, we reanalyzed UT *A. pleuropneumoniae* isolates using rapid slide agglutination (RSA) test and multiplex PCR.

We examined 47 isolates obtained from porcine pleuropneumonic lesions submitted for routine diagnosis to the Livestock Hygiene Service centers in Japan between 2003 and 2013. Forty-seven isolates were UT based on the AGD test using antigens extracted by autoclaving [16]. These isolates were defined as serovar UT.

Forty-seven isolates were investigated by the RSA test using antisera against serovars 1–15, as previously described [12]. Twenty-five isolates reacted with antiserum against serovar 1 (UT-serovar 1; Table 1). Nineteen isolates reacted

with antiserum against serovar 2 (UT-serovar 2). One isolate reacted with antiserum against serovar 15 (UT-serovar 15). Two isolates reacted with multiple antisera and were UT.

Forty-seven isolates were serotyped by multiplex PCR as previously described by Ito [8]. Twenty-four and 18 isolates showed the patterns of serovars 1 and 2, respectively (Table 1). Five isolates were UT using this method. The 5 isolates that were UT by multiplex PCR, as described by Ito [8], were then serotyped by multiplex PCR, as described by Bossé *et al.* [2]. One and 2 isolates showed the patterns of serovars 1 and 2, respectively. Two isolates were UT using this method. Subsequently, the two isolates that were UT by multiplex PCR, as described by Bossé *et al.* [2], were serotyped by multiplex PCR, as described by Turni *et al.* [17]. These 2 isolates showed the pattern of serovar 15. Therefore, it was possible to determine the serovars of isolates UT by the AGD test using the RSA test and multiplex PCR. The serovars of isolates identified as serovars 1, 2 or 15 by the RSA test were confirmed by the results of serotyping by multiplex PCR.

The autoclaved antigens (121°C, 1 hr) used in the AGD test have a higher specificity than heat-extracted antigens (60°C, 2 hr) [16]. To investigate why identification of the serovars of these isolates was impossible using the AGD test, the antigens extracted by heat were used in place of autoclaved antigens. Thirty-seven isolates were used in this test (UT-serovar 1: 23 isolates; UT-serovar 2: 13 isolates; and UT-serovar 15: one isolate). Precipitation lines were observed for the heat-extracted antigens of all UT-serovar 1 and UT-serovar 15 isolates, but were not observed for any UT-serovar 2 isolates (Fig. 1). To confirm the influence of temperature on antigen extraction, we conducted the RSA test using antigens derived by both autoclaving and heat extraction. UT-Serovar 2 isolates exhibited the same reaction regardless of antigen-extraction temperature, whereas serovar 1 isolates showed only a weak reaction using antigens extracted at 100°C and 121°C (Fig. 2). Stronger heat treat-

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Table 1. Serotyping reanalysis of unserotypable *Actinobacillus pleuropneumoniae* isolates by an agar gel diffusion test

	serovar			
	UT	1	2	15
RSA test	2 <sup>a)</sup>	25	19	1
multiplex PCR	0	25	20	2
method by Ito (serovars 1, 2 and 5) <sup>b)</sup>	5	24	18	-
method by Bosse <i>et al.</i> (serovars 1-3, 5-8, 10 and 12)	2 <sup>c)</sup>	1 <sup>c)</sup>	2 <sup>c)</sup>	-
method by Turni <i>et al.</i> (serovar 15)	-	-	-	2 <sup>d)</sup>

a) Number of isolates, b) The serovars that we investigated, c) Unserotypable isolates using multiplex PCR by Ito, d) Unserotypable isolates using multiplex PCR by Bosse *et al.*

ment affects the thermostable antigens used for serotyping in *Haemophilus parasuis* [3]. Likewise, our findings suggested that the serotyping antigens of serovars 1 and 15 were affected by heat. Antigens used in the AGD test are supernal and are autoclaved and centrifuged [16]. UT-serovar 2 isolates were serotypable using the RSA test, but remained UT by the AGD test even when using antigens derived at different extraction temperatures. It was impossible to extract the serotyping antigen of serovar 2 by autoclaving or heat treatment. Therefore, it is necessary to select a serotyping method in consideration of the characteristics of each serovar.

To investigate the relationship between antigenic differences and the genotype of isolates UT by the AGD test, we performed pulsed-field gel electrophoresis (PFGE) analysis as previously described [14]. Six isolates (15-PLA-19, 17-PLA-15, 17-PLA-18, 17-PLA-25, 17-PLA-39 and 17-PLA-43) that were typed serovar 2 and one isolate (15-PLA-22) that was typed serovar 15 by the RSA test and multiplex PCR were investigated. The PFGE profiles were analyzed using the software Molecular Analyst Fingerprinting Plus (version 1.6; Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), with CHEF DNA Size standard lambda Ladder (Bio-Rad Laboratories) as the gold standard. 17-PLA-45 (serovar 2), *A. pleuropneumoniae* HS143 (serovar 15 reference strain), 15-PLA-20 (serovar 15) and 16-PLA-6 (serovar 15) were used as controls. The restriction enzyme digest patterns following DNA digestion with *ApaI* are shown in Fig. 3. Serovar 2

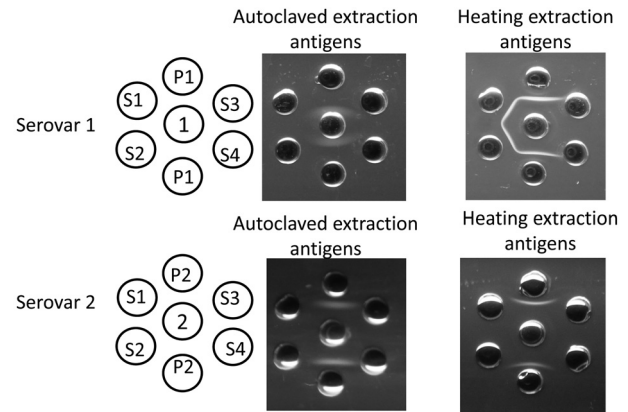


Fig. 1. AGD test using autoclaved extraction antigens or heating extraction antigens. 1: antiserum of serovar 1, 2: antiserum serovar 2, P1: antigen of *A. pleuropneumoniae* 4074 (serovar 1 reference strain), P2: antigen of *A. pleuropneumoniae* 1536 (serovar 2 reference strain), S1 and S2: antigens of 23-PLA-35 and 23-PLA-36, respectively (determined serovar 1 by RSA test), S3 and S4: antigens of 23-PLA-52 and 23-PLA-53, respectively (determined serovar 2 by RSA test).

isolates exhibited three PFGE patterns. Four isolates showed the same pattern as the 17-PLA-45, but two differed. Several different PFGE patterns existed among the UT-serovar 2 isolates. Approximately 80% of serovar 2 field isolates in Japan show the same PFGE pattern as 17-PLA-45 [14]. The predominant PFGE pattern of UT-serovar 2 was identical to that of serovar 2, suggesting that the antigenic differences between serovar 2 and UT-serovar 2 were not reflected by their PFGE patterns. Thus, it is necessary to perform other genetic analyses to investigate the relationship between antigenicity and genotype. For serovar 15, the PFGE pattern of 15-PLA-22 differed from those of the reference strain and serovar 15 field isolates. It was confirmed that the genotype of 15-PLA-22 was different from those of serovar 15. As the number of isolates used in this study was small, it is unclear whether the antigenic differences between serovar 15 and UT-serovar 15 are reflected by their PFGE patterns. It is necessary to increase the number of isolates to investigate

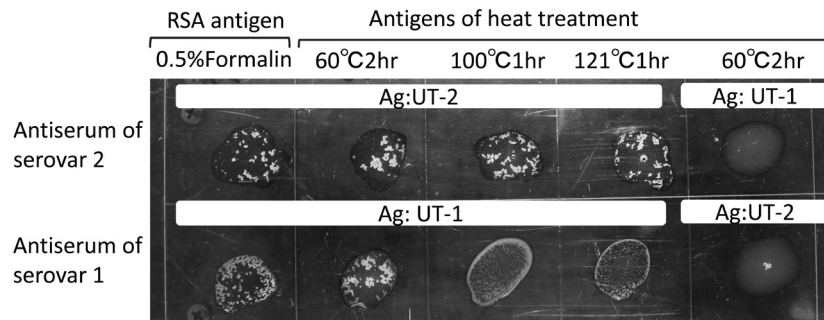


Fig. 2. RSA test with heat treatment antigens. Ag: antigen, UT-1: isolate of UT by AGD test but serovar 1 by RSA test, UT-2: isolates of UT by AGD test but serovar 2 by RSA test.

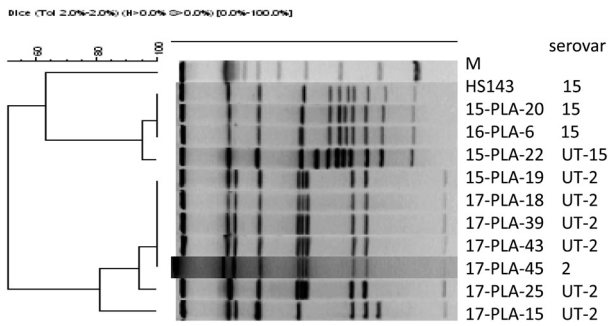


Fig. 3. PFGE patterns of serovar 2 isolates and serovar 15 isolates. M: Lambda ladder, HS143: reference strain of serovar 15, 15-PLA-20 and 16-PLA-6: serovar 15, 15-PLA-22: determined serovar 15 by multiplex PCR, 15-PLA-19, 17-PLA-15, 17-PLA-18, 17-PLA-25, 17-PLA-39 and 17-PLA-43: determined serovar 2 by RSA test and multiplex PCR, 17-PLA-45: serovar 2.

the relationship between antigenicity and genotype.

In this study, 47 isolates UT using the AGD test were investigated using the RSA test and multiplex PCR. As a result, it was possible to identify 25, 20 and two isolates as serovars 1, 2 and 15, respectively. The AGD test and the RSA test using antiserum could confirm expression of serovar-specific antigen and difference of the antigenicity. Our investigation suggested that serovar-specific antigens from some *A. pleuropneumoniae* isolates of serovars 1 and 15 may be easily denatured by heat treatments, such as 100°C for 1 hr, whereas some of serovar 2 isolates may be hard to extract by heat treatments, such as 121°C for 1 hr. These facts should give consideration to preparation of antigen for AGD test. The results of multiplex PCR based on serovar-specific gene had agreed with that of the RSA test in our study. Therefore, it would suggest that the multiplex PCR methods are useful methods for serotyping as rapid, simple and precise diagnosis tools. To identify the factor that causes difference of extraction of the antigen by heat treatment, further genetic analyses are necessary.

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