NOTE *Bacteriology*

Isolation of Atypical Genotype *Actinobacillus pleuropneumoniae* Serotype 6 in Japan

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ABSTRACT. We describe here isolation of genetically atypical serotype 6 *Actinobacillus pleuropneumoniae* in Japan indistinguishable by the multiplex PCR that can discriminate between immunologically cross-reactive serotypes 3, 6 and 8. Nucleotide sequence analysis of capsular export and biosynthesis genes revealed that the atypical isolates have capsular polysaccharide export and synthesis gene sequences that are distinct from those of the serotype 6 reference strain. The atypical strains contain a sequence that is identical with both serotype 3- and 6-specific primers, which causes cross-reactions in multiplex PCR.

KEY WORDS: *Actinobacillus pleuropneumoniae*, atypical serotype 6, capsular polysaccharide export and biosynthesis genes, multiplex PCR, nucleotide sequence.

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Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, which causes serious economic losses in the pig-rearing industry [4]. To date, 15 serotypes have been recognized mainly based on the antigenic diversity of capsular polysaccharides in the organisms [2, 18]. The prevalent serotypes differ by country, region and farm [3]. In Japan, serotype 2 is the most common; serotypes 1 or 5 follow [3, 7, 15, 16, 22]. Serotypes 3, 6, 7, 8, 9, 11, 12 and 15 are isolated in only sporadic cases [3, 7]. Serotyping is widely performed in veterinary diagnostic laboratories, since virulence differs in serotypes and vaccines for *A. pleuropneumoniae* are serotype specific [4, 17, 20]. However, only a few veterinary diagnostic laboratories can prepare a full set of serotype-specific antisera for serotyping. Furthermore, cross-reactions are often observed among different serotypes in a number of conventional immunological tests, for example, between serotypes 1, 9 and 11, serotypes 4 and 7 and between serotypes 3, 6 and 8, which prevent accurate and rapid typing of field strains [11–14].

To overcome such problems, genotypic methods, such as PCR amplification of serotype-specific capsular biosynthesis or export genes, have been developed to enable precise serotyping [1, 7, 8, 10, 19, 23, 24]. Among them, a specific multiplex PCR that can discriminate among the problematic cross-reactive serotype 3, 6 and 8 strains in a single tube has been developed [24]. Recently, we isolated two serotype 6 strains from different prefectures with an immunodiffusion test. Then, we tried to genetically confirm them as serotype 6 by the single-tube multiplex PCR [24]. However, the Japanese isolates could not be typed with the multiplex PCR due to cross-reaction. In this study, we report serological and genetic characterization of the atypical Japanese *A. pleuropneumoniae* serotype 6 isolates.

An *A. pleuropneumoniae* designated as strain QAS59 was isolated from an abscess of a pig's backbone in Fukui, Japan, in 2009, and another strain designated as HYT2 was isolated from an abscess of a pig's lung at an abattoir in Tochigi, Japan in 2010. They were grown at 37°C in 5% CO_2 on chocolate agar or tryptic soy agar supplemented with 5% horse blood and 50μg/m*l* nicotinamide dinucleotide as previously described [7].

Serotyping was carried out by slide agglutination and immunodiffusion tests using rabbit hyperimmune sera against reference strains of the 15 serotypes of *A. pleuropneumoniae* [13, 14]. The results are shown in Table 1. The two Japanese isolates were serotyped as serotype 6 by the immunodiffusion test, although these strains showed cross-reactivity against more than two antisera against serotype reference strains by the slide agglutination test.

Genotyping was then carried out by a multiplex PCR that can distinguish between serotypes 3, 6 and 8 based on the *cps* and/or *cpx* gene [24], a PCR typing system based on the *A. pleuropneumoniae* toxin (Apx toxin) and outer membrane lipoprotein genes (*apx* and *omlA*, respectively) [5]. In addition, a monoplex PCR with either serotype 3-, 6- or 8-specific primer sets only was also performed. Results for the genotyping are shown in Table 1 and Fig. 1. In the multiplex PCR, both Japanese strains had two amplicons (Table 1, Fig. 1A and 1C). Therefore, we also tried a monoplex PCR with only serotype 3-, 6- or 8-specific primers. In these experiments, each Japanese strain had an amplicons in each capsular serotype 3 and 6 monoplex PCR (Table 1, Fig. 1B and 1C). Apx-toxin gene profiling resulted in the same *apx* gene combination as the serotype 4, 6, 8 and 15

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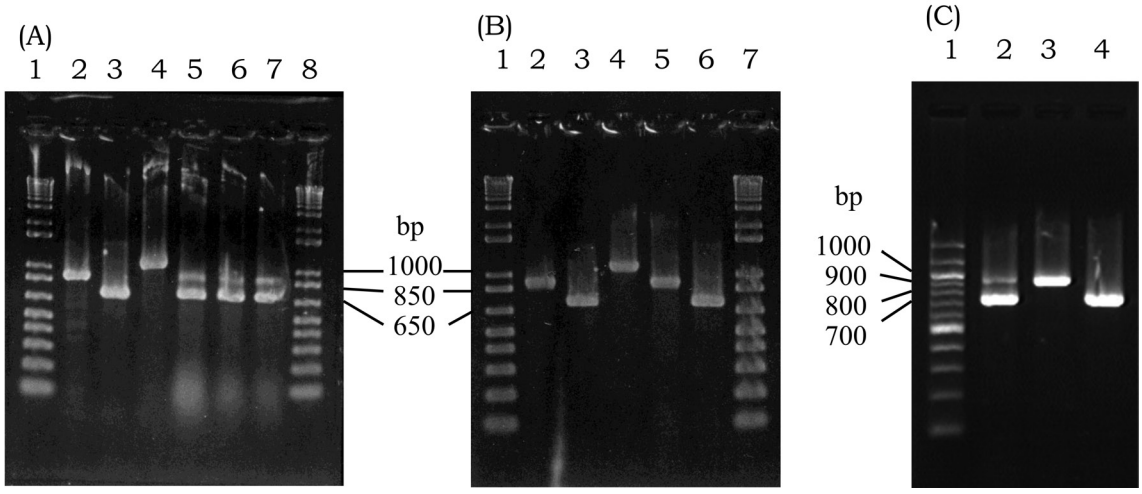

Fig. 1. (A) Serotype 3, 6 and 8 multiplex PCR for strain HYT2. Lanes 1 and 8, DNA size marker (1 kb plus DNA ladder, Invitrogen); lane 2, serotype 3 reference strain; lane 3, serotype 6 reference strain; lane 4, serotype 8 reference strain; lanes 5 to 7, atypical serotype 6 strain HYT2. The sizes of some DNA fragments included in the DNA size marker are shown on the right. (B) Serotype 3 and 6 monoplex PCR for strain HYT2. Lanes 1 and 7, DNA size marker (1 kb plus DNA ladder, Invitrogen); lane 2, serotype 3 reference strain; lane 3, serotype 6 reference strain; lane 4, serotype 8 reference strain; lane 5, atypical serotype 6 strain HYT2 in the serotype 3 monoplex PCR; lane 6, atypical serotype 6 strain HYT2 in the serotype 6 monoplex PCR. The sizes of some DNA fragments included in the DNA size marker are shown on the left. (C) Multiplex and monoplex PCR for strain QAS59. Lane 1, DNA size marker (100 bp DNA ladder, New England Biolabs); lane 2, atypical serotype 6 strain QAS59 in the serotype 3, 6, and 8 multiplex PCR; lane 3, atypical serotype 6 strain QAS59 in the serotype 3 monoplex PCR; lane 4, atypical serotype 6 strain QAS59 in the serotype 6 monoplex PCR. The sizes of some DNA fragments included in the DNA size marker are shown on the left.

Table 1. Serotyping and genotyping of atypical Japanese *A. pleuropneumoniae* isolates

Tests	Strains			
	Serotype 3 reference strain	Serotype 6 reference strain	Strain QAS59	Strain HYT2
Serotyping tests				
Slide agglutination test	NT[a)]	NT	3, 6, 8, 15	6, 10, 11, 15
Immunodiffusion test	3[b)]	6	6	6
Genotyping tests				
Multiplex PCR				
Serotypes 3, 6 and 8	3	6	3, 6	3, 6
Monoplex PCR				
Serotype 3	+	–	+	+
Serotype 6	–	+	+	+
Serotype 8	–	–	–	–
Apx toxin PCR profiling				
apxICA	–	–	–	–
apxIICA	+	+	+	+
apxIIICA	+	+	+	+
apxIBD	–	+	–	+
apxIIIBD	+	+	+	+
omlA typing	*omlAIII*	*omlAIII*	*omlAIII*	*omlAIII*

a) Not tested. b) The numbers indicate the positive serotypes.

reference strains in strain HYT2, while the same apx gene combination was seen in strain QAS59 as in the serotype 3 reference strain (Table 1) [5]. The *omlA* gene typing scheme for both Japanese strains showed an *omlAIII* pattern, which was the same as that found for the serotype 3, 6 and 7 reference strains (Table 1) [5].

The ultimate goal in this study was to clarify why two amplicons were observed in the Japanese serotype 6 isolates with the multiplex PCR. We speculated that the Japanese serotype 6 isolates might have possessed identical or highly

similar sequences with serotype 3-specific primers (APN3F, 5′-TTT GCG CTG TAG TGC TCC AAT-3′, and APN3R, 5′-AAC AAA TAA AGT TGC TCG AAA GTA) in the multiplex PCR [24], resulting in cross-reaction in the multiplex PCR. Therefore, the nucleotide sequences of the *cpxD-cpsA* region, in which the serotype 6-specific PCR primers for the multiplex PCR [24] are located, of the Japanese isolates were determined. The DNA regions were PCR amplified, purified and sequenced as previously described [6]. The primers used for amplification of the DNA region (5′-CAC ACG ATA AAC CGT TGG TAC ATC-3′ and 5′-GTG ATC GCC TAA TGA TCT TGC TCT TTC-3′) were designed in this study based on the *cpx6D* and *cps6A* sequence (accession number (no.) AY534316) [9]. The nucleotide sequences (2,410 base pairs (bp)) of the *cpxD-cpsA* region of QAS59 and HYT2 were identical and deposited into the DDBJ/GenBank/EMBL databases under accession no. AB809625. As we expected, QAS59 and HYT2 had sequences identical to serotype 3-specific primers APN3F and APN3R as well as serotype 6-specific primers, resulting in amplification of two amplicons from both serotypes 3- and 6-specific primer sets.

The nucleotide sequences of the entire *cpsA* and partial *cpxD* genes of the Japanese isolates were then compared with nucleotide sequences in databases. Consequently, the entire nucleotide sequences of the *cps6A* gene (1,137 bp) of QAS59 and HYT2 showed highest identity with that of *A. pleuropneumoniae* serotype 7 (97.3%, accession no. CP001091) followed by those of serotypes 8 (96.9%, accession no. AY3565527), 6 (92.9%, accession no. AY534316) and 3 and 2 (91.3%, accession no. CP000687 and AY377726, respectively). A high degree of homology between *cpsA* genes, which is involved in capsular biosynthesis of *A. pleuropneumoniae* serotypes 2, 3, 6, 7 and 8, has already been reported [9], indicating a similar function for CpsA encoded by *cpsA* genes in the five serotypes [9]. It has also been suggested that genes other than *cpsA* genes determine the capsular antigenic differences among these serotypes [9, 21]. For example, *cps6E* [9, 21] and *cps6F* [21] were present only in serotype 6. It is of interest that the *cps6A* genes of strains QAS59 and HYT2 did not show the highest identity with those of the serotype 6 reference strains despite the fact that we expected that the CPS synthesis genes were conserved in the serotype. Furthermore, the partial nucleotide sequence of the *cpxD* gene (993 bp), which is not involved in capsular antigenicity but is involved in capsular export, of the Japanese isolates revealed the highest similarity (97.2%, accession no. AY534316) with that of the serotype 6 reference strain, followed by serotype 2 (95.5%, accession no. AY377726); serotype 7, 3 and 1 (94.9%, accession no. CP001091, CP000687 and AF518558, respectively); serotype 12 (91.5%, accession no. AY496881); serotype 4 (91.1%, accession no. GU585380); and serotype 5 (89.4%, accession no. CP000569), indicating that the capsular export gene, *cpxD,* is conserved more than the capsular synthesis gene, *cpsA,* in serotype 6 strains.

In conclusion, two Japanese serotype 6 *A. pleuropneumoniae* isolates are genetically distinct from those isolated from countries other than Japan [24] due to unique *cpxD* and *cpsA* genes, resulting in the inability to use multiplex PCR for serotypes 3, 6 and 8 *A. pleuropneumoniae* [24]. Development of an alternative typing method is needed for genetically atypical serotype 6 *A. pleuropneumoniae* isolates.

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