

REVIEW

Development of Typing Methods of *Actinobacillus pleuropneumoniae* Based on the Antigenic and Genetic Diversity of the Protective Outer Membrane Lipoprotein

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Abstract

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia that is one of the important bacterial diseases of the respiratory tract of pigs. The disease occurs worldwide and results in serious economic losses to the pig-rearing industry. In this review, new typing methods based on the antigenic and genetic diversity of a protective outer membrane lipoprotein (OmlA), that have been developed for control and epidemiological study of the disease, are described. First, the gene for the OmlA of an *A. pleuropneumoniae* field isolate (serotype 5a) has been cloned and characterized. The lipoprotein has been shown to be a protective antigen of the organism. Southern blot and the nucleotide sequence analysis of the gene for the lipoprotein have revealed the presence and diversity of the gene in *A. pleuropneumoniae*. Secondly, based on the antigenic diversity of OmlA, Western blot analysis with specific antisera against three antigenically distinct OmlAs has been developed. The antisera can differentiate *A. pleuropneumoniae* into three groups. Finally, based on the genetic diversity of OmlA, a PCR-Restriction Fragment Length Polymorphism (RFLP) typing method has been developed. This method can divide *A. pleuropneumoniae* into five groups. The grouping results by the Western blot analysis were correlated with that by the PCR-RFLP typing method. In addition, the PCR-RFLP typing method can be performed in almost all veterinary diagnostic laboratories since the method does not require specific antisera and expensive reagents and apparatus. Therefore, the PCR-RFLP typing method can be substituted for other OmlA typing and seems to be the most convenient and practical OmlA typing method at present. The new typing methods developed in this study should be used as tools which are effective and practical for control of porcine pleuropneumonia caused by *A. pleuropneumoniae*.

Discipline: Animal Health

Additional key words: PCR-RFLP, Porcine pleuropneumonia, Western blot analysis

Introduction

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia that is characterized by a fibrinous-hemorrhagic pneumonia and is one of the important bacterial diseases of the respiratory tract of pigs³⁶. To date, 15 serotypes are recognized based on an-

tigenic diversity of capsular polysaccharide and lipopolysaccharide^{3,31}. The disease occurs worldwide and results in serious economic losses to the pig-rearing industry³⁶. Prevalent serotypes differ according to country^{1,3,7,10,29,37,39}, area and farm.

At present polyclonal antisera raised against whole bacterial cells of *A. pleuropneumoniae* are utilized for serotyping^{20,21,26,27}. However, only a few veterinary diag-

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nostic 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 the methods, which prevent accurate typing^{12,22,23,24,25,28}. The cross-reactions are most likely due to the fact that the polyclonal antisera contain antibodies directed against a variety of antigens including antigens common in *A. pleuropneumoniae*. In addition, quantitative differences of expressed antigens including both serotype-specific and cross-reacting antigens may affect the results of typing. For these reasons, the development of a new typing method that is not affected by cross-reacting antigens and is useful for prevention of porcine pleuropneumonia is desirable. Therefore, it seems that development of a new typing method based on a particular single antigen and gene would facilitate a more precise typing of *A. pleuropneumoniae* isolates without influence from the cross-reacting antigens.

Furthermore, if the antigen for typing were a protective antigen of *A. pleuropneumoniae*, the new typing method could be used as a tool which is directly effective and practical for control and epidemiological analysis of the disease. Especially, to know the antigenic type of protective antigens is important for the appropriate control of porcine pleuropneumonia by vaccination.

This review is part of a series of studies conducted to develop a new typing method based on a particular protective antigen (an outer membrane lipoprotein (OmlA protein)) and its gene (*omlA* gene) of *A. pleuropneumoniae*. The usefulness and importance of the OmlA protein for the prevention of *A. pleuropneumoniae* infection^{4,11,16} are also manifest by the fact that a recombinant vaccine that contains the OmlA protein as one of protective components is licensed by a vaccine company in North America². The usefulness and importance of the *omlA* gene for diagnosis of *A. pleuropneumoniae* infection are also proved by other researchers' reports^{5,8,13,14,32,33}. Currently a diagnostic kit for detection and typing of *A. pleuropneumoniae* using the *omlA*-PCR is marketed by a company in France^{8,32}. Species specificity of a number of the *omlA*-PCR systems has also been examined, and they have been shown to be species-specific^{13,32,33,38}.

Cloning and characterization of an outer membrane lipoprotein of an *Actinobacillus pleuropneumoniae* serotype 5a field isolate¹⁶

The gene encoding an OmlA protein with a molecular mass of 43–kDa was cloned from a Japanese field isolate of *Actinobacillus pleuropneumoniae* (strain NG-8, serotype 5a)¹⁶. Immunization of the OmlA protein protected mice from death in a challenge experiment with an

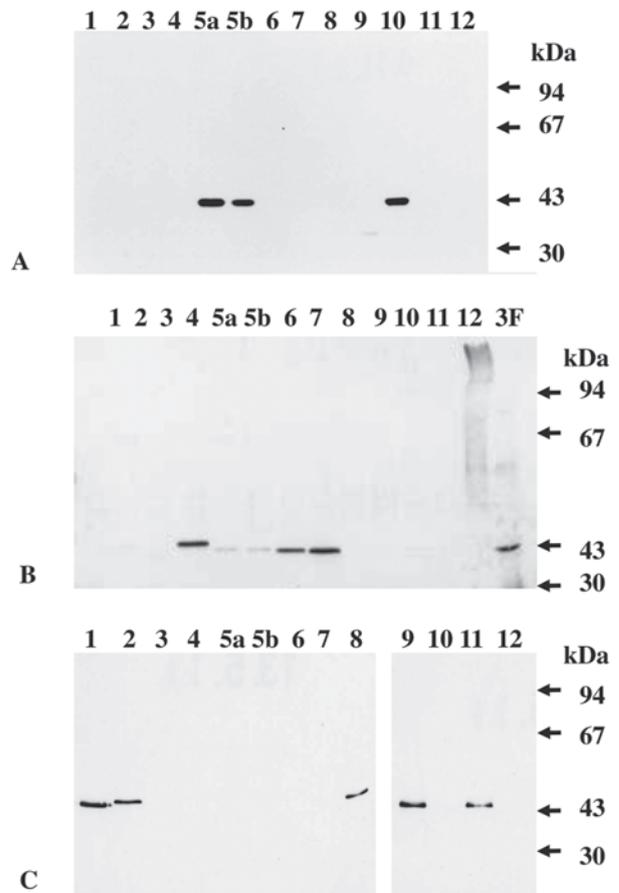


Fig. 1. Western blot of whole-cell lysates of *A. pleuropneumoniae* serotype reference and field strains

The top blot (A) was probed with the antiserum against the OmlA protein of an *A. pleuropneumoniae* field isolate (NG-8, serotype 5a). The middle blot (B) was probed with the antiserum against the OmlA protein of an *A. pleuropneumoniae* reference strain (WF83, serotype 7). The bottom blot (C) was probed with the antiserum against the OmlA protein of an *A. pleuropneumoniae* reference strain (serotype 1). Numbers above each lane indicate the serotype of the reference strain. 3F indicates the field strain of serotype 3. Molecular mass markers are shown on the right (in kDa).

A. pleuropneumoniae serotype 5, indicating that OmlA protein is a protective antigen of *A. pleuropneumoniae*¹⁶. The protective efficacy of the OmlA protein from *A. pleuropneumoniae* serotype 5 has been supported by a challenge experiment with pigs by other researchers⁴. Southern blot analysis under low-stringency conditions revealed the presence of a hybridizing sequence in reference strains of serotypes 1, 2, 3, 4, 5a, 5b, 6, 7, 8, 9, 10, 11, and 12, which are prevalent serotypes in Japan^{1,10,37,39} except for serotypes 4 and 10, indicating the presence of allelic variants of *omlA* gene in the strains of these sero-

types¹⁶. While, Southern blot analysis under high-stringency conditions showed the presence of a sequence highly homologous to the *omlA* gene of strain NG-8 in reference strains of serotypes 5a, 5b and 10¹⁶. In addition, a specific serum against OmlA protein of NG-8 detected a homologous protein in the strains of serotypes 5a, 5b and 10 (Fig. 1A), suggesting that these serotypes carry an *omlA* gene and an OmlA protein which are each highly similar among the serotypes. From the data obtained by Western (Fig. 1A) and Southern blot analysis¹⁶, the presence of antigenic and genetic diversity of an *A. pleuropneumoniae* OmlA protein was indicated. In conjunction with a protective efficacy of OmlA protein, general presence of *omlA* gene in *A. pleuropneumoniae* and its potential genetic diversity indicate that the OmlA protein and its gene can be used for typing of *A. pleuropneumoniae* which is directly effective and practical for appropriate control of porcine pleuropneumonia caused by *A. pleuropneumoniae*.

Genetic diversity of the outer membrane lipoprotein

Nucleotide sequence determination of DNA containing the *omlA*-like gene in reference strains of *A. pleuropneumoniae* serotype 1 to 12 (the accession number: AB007572 to AB007584, respectively) revealed that the *omlA* gene generally exists in *A. pleuropneumoniae* isolates. The genetic diversity of the *omlA* gene and the OmlA protein among *A. pleuropneumoniae* serotypes has been exhibited by the comparison of the gene (Table 1). The positions of the conserved and variable sequences of the *omlA* gene among *A. pleuropneumoniae* serotypes could be identified. The nucleotide sequence-based typing of the *omlA* genes could be used to differentiate *A. pleuropneumoniae* isolates since the phylogenetic analysis of the nucleotide sequence of *omlA* genes (Fig. 2 and Table 2) divided *A. pleuropneumoniae* serotypes into four groups: Group I, serotype 1, 9, 11, and 12; Group II, sero-

Table 1. Percentage identities in nucleotide sequence between the *omlA* gene of reference strains of *A. pleuropneumoniae* serotypes 1 to 12

Gene	% pairwise identities in nucleotide sequences			
	Group I	Group II	Group III	Group IV
Group I ^{a)} Serotype 1, 9 and 11	99.3 to 100	77.5 to 82.2	63.0 to 75.6	63.3 to 72.5
Group II ^{a)} Serotype 2 and 8		100	70.7 to 73.6	74.2 to 74.5
Group III ^{a)} Serotype 3, 4, 6, and 7			95.4 to 99.9	69.5 to 79.1
Group IV ^{a)} Serotype 5a, 5b and 10				99.6 to 99.8

a): See Fig. 2.

Table 2. Grouping based on the antigenic and genetic diversity of the OmlA protein and the *omlA* gene

Grouping by the Western blot analysis ^{a)}		Grouping by the nucleotide sequencing analysis ^{b)}		Grouping by the PCR-RFLP analysis ^{c)}	
Antigenic group	Serotype	Sequence group	Serotype	PCR-RFLP group	Serotype
A	1, 2, 8, 9, and 11	I	1, 9, 11, and 12	I	1, 9, 11, and 12
		II	2 and 8	II	2 and 8
B	3 ^{d)} , 4, 6, and 7	III	3, 4, 6, and 7	III	3, 6 and 7
		IV		IV	4
C	5a, 5b and 10	IV	5a, 5b and 10	V	5a, 5b and 10

a): Antigenic typing method developed in this study. Serotype 12 strain cannot be grouped into any group since it did not express any detectable OmlA protein reacting with the antisera prepared in this study.

b): Genetic typing method developed in this study.

c): Genetic typing method developed in this study.

d): A field isolate of *A. pleuropneumoniae* serotype 3 belongs to Group B (Fig. 1). While serotype 3 reference strain did not express any detectable OmlA protein reacting with the antisera prepared in this study.

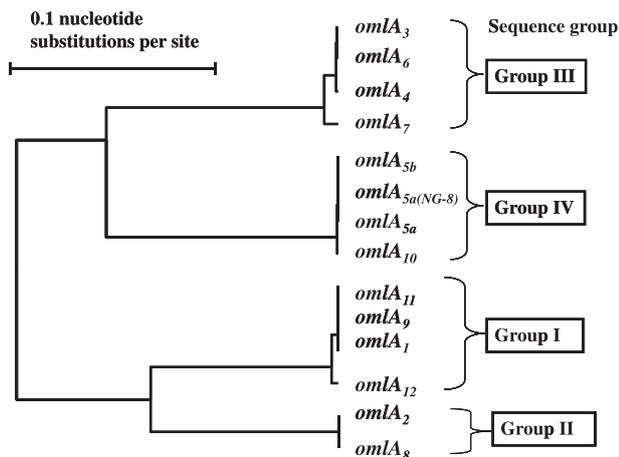


Fig. 2. Phylogenetic tree based on the nucleotide sequence conducted by UPGMA of Genetyx version 7
Number following "omlA" indicates the serotype.

type 2 and 8; Group III, serotype 3, 4, 6, and 7; and Group IV, serotype 5a, 5b and 10. Since a DNA sequencer and reagents for nucleotide sequence determination are extremely expensive, only a very few veterinary diagnostic laboratories can determine the nucleotide sequence of the *omlA* gene by themselves. Therefore we have subsequently developed typing methods that are less expensive and can be performed without expensive apparatus in standard veterinary diagnostic laboratories such as Livestock Hygiene Centers in Japan.

Development of typing method based on the antigenic diversity of the outer membrane lipoprotein^{16,17}

A new serotyping method using Western blot analysis with specific antisera against three antigenically distinct OmlA proteins has been developed^{16,17}. The Western blot analysis using specific antisera against OmlA_{5a(NG-8)} (Fig. 1A), OmlA₇ (Fig. 1B) and OmlA₁ (Fig. 1C) could differentiate reference strains of *A. pleuropneumoniae* serotypes 1, 2, 4, 5a, 5b, 6, 7, 8, 9, 10, and 11 into three groups: Group A, serotypes 1, 2, 8, 9, and 11; Group B, serotypes 4, 6 and 7; Group C, serotypes 5a, 5b and 10 (Table 2). The reference strain of *A. pleuropneumoniae* serotype 3 and 12 did not express any detectable OmlA protein (Fig. 1 and Table 2). However, a field isolate of *A. pleuropneumoniae* serotype 3 could be grouped into Group B (Fig. 1B and Table 2). Regarding serotype 12 reference strain, Gerlach et al.¹¹ observed a different result in Western blot analysis using a specific antiserum against the OmlA protein prepared from serotype 1 strain. They demonstrated that serum against the OmlA protein from a serotype 1 field isolate could detect an immuno-

logically homologous protein in serotype 12 reference strain. An ORF (accession numbers: AB007574, AB007584) that has a sequence highly homologous to the *omlA*₇ and *omlA*₁ gene is present in serotypes 3 and 12 reference strains stocked in our laboratory, respectively. Hence, serotypes 3 and 12 reference strains used in this study may not produce OmlA protein due to a genetic mutation in the region other than an ORF during repeated passages in laboratories. The three OmlA groups (groups A to C) by Western blot analysis could be distinguished by the nucleotide sequence-based typing (Table 2).

Development of typing method based on the genetic diversity of the outer membrane lipoprotein³⁰

A new genetic typing method using PCR-RFLP based on genetic diversity of the *A. pleuropneumoniae* *omlA* gene was developed³⁰. Primers for PCR that amplify all *A. pleuropneumoniae* strains (5'-CCTAAGGTGATATGTCCGCA-3' and 5'-GTTGCCGTAGCACCGATTACG-3') were selected from a conserved sequence of the *omlA* of *A. pleuropneumoniae*. A DNA fragment of approximately 970 bp was amplified from the genomic DNA of 41 Japanese (serotypes 1, 2, 3, 5, and 7) and 1 Canadian (serotype 10) field isolates of *A. pleuropneumoniae* as well as that of reference strains of *A. pleuropneumoniae* serotypes 1 to 12 (data not shown), implying that the *omlA* gene is generally present in *A. pleuropneumoniae* field isolates as well as reference strains stocked in laboratories. Digestion of the DNA amplified from the reference and field strains with the enzymes *VspI* yielded specific polymorphic patterns, showing that the *omlA* genotype is inherent to a given serotype and allowing discrimination of all serotypes into five distinct groups: Group I, serotype 1, 9, 11, and 12; Group II, serotype 2 and 8; Group III, serotype 3, 6 and 7; Group IV, serotype 4; and Group V, serotype 5a, 5b and 10 (Fig. 3 and Table 2). Antigenic type of three OmlA protein groups by Western blot analysis (groups A to C) could be substituted by the PCR-RFLP typing since the antigenic groups could be predicted by the PCR-RFLP grouping (Table 2). OmlA serotyping with Western blot analysis may be the best typing method for examination of expression and antigenicity of the OmlA for vaccination. However, the PCR-RFLP typing seems to be more useful for epidemiological study since the PCR-RFLP typing has more superior discriminatory ability than the Western blot analysis. Since the specific antisera for OmlA serotyping are not easily available in veterinary diagnostic laboratories, popularization of the OmlA serotyping may not be easy. On the contrary, the specific antisera are not required for

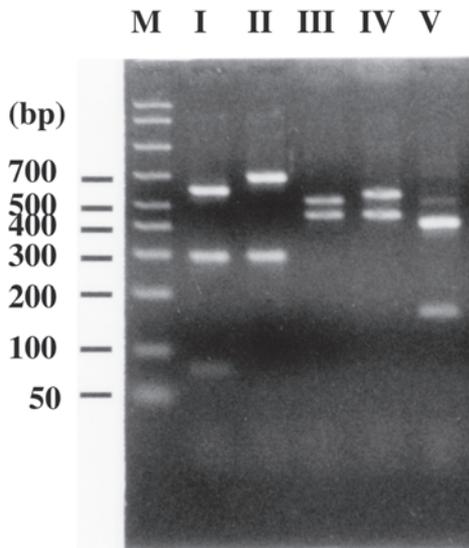


Fig. 3. The PCR-RFLP profiles of each groups after digestion with the restriction enzyme *VspI*
DNA size marker, M, is shown on the left. Roman numerals above each lane correspond to the PCR-RFLP group.

the PCR-RFLP typing and all reagents for the analysis are commercially available. Therefore, standard veterinary diagnostic laboratories equipped with PCR apparatus can easily do the OmlA typing by the PCR-RFLP typing method instead of OmlA serotyping by Western blot analysis.

To date, several PCR-RFLP-based or PCR-based typing methods using allelic variation of the target gene have been proposed for typing of *A. pleuropneumoniae*. The PCR-RFLP methods using the *aroA* gene encoding 5-enolpyruvylshikimate-3-phosphatase (a key enzyme in the biosynthesis of aromatic amino acids)¹⁵ and *apxIV* gene encoding an exotoxin (ApxIV) of *A. pleuropneumoniae*¹⁸ have been developed for typing of the organism. However, it is unknown whether PCR tests for typing of the genes are protective antigens or not. Multiplex PCR tests for typing of the genes involved in the biosynthesis of capsular polysaccharides of *A. pleuropneumoniae* serotypes 2, 5 and 6¹⁹ and serotypes 1, 2 and 8³⁴ have also been developed. However, these tests cannot type *A. pleuropneumoniae* isolates other than serotypes 1, 2, 5, 6, and 8.

Regarding typing methods whose target DNA is a gene for protective antigens of *A. pleuropneumoniae* other than the *omlA* gene, PCR assays based on the *apx* genes encoding toxins (ApxI, ApxII and ApxIII toxins) and a PCR-RFLP test based on the *tbpB* gene encoding transferring-binding protein (TbpB) which is a component of the iron-uptake system of *A. pleuropneumoniae* have been developed^{6,9,14,35}. Multilocus typing of such genes

encoding the protective antigens together with the *omlA* gene developed in this study would provide more effective and practical information on control and epidemiological analysis of porcine pleuropneumonia.

Consequently, the new genetic typing method of a protective OmlA protein developed in this study can be used as a routine tool which should be directly effective and practical for control and epidemiological analysis of porcine pleuropneumonia in veterinary diagnostic laboratories.

Conclusion

To examine antigenic type of *A. pleuropneumoniae* protective antigens is important for choice of vaccine. Therefore the genetic and antigenic typing methods of the OmlA protein described in this review used either alone or in combination should be directly effective and practical tools for control and epidemiological analysis of porcine pleuropneumonia caused by *A. pleuropneumoniae*.

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