

Intra-serovar differentiation and evaluation of serovar 7 *Actinobacillus pleuropneumoniae* field strains using PCR and Western blot assays

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Introduction

Actinobacillus pleuropneumoniae (App) is the etiologic agent of porcine pleuropneumonia that can cause serious losses to pig industry worldwide due to death loss of grower and finishing pigs. App 1, 5, and 7 are the most commonly isolated serovars in this country. Of them, App serovar 7 has been emerging as an important pathogen during the past few years.¹ In fact, about 60% of the App strains that were isolated in our diagnostic laboratory during the past year were serovar 7. The reason is not known. So far a wide variety of serologic tests have been developed. However, cross-reactivity between strains of various App serovars and other bacterial species remains problematic. For this reason, several PCR-RFLP-based typing methods using allelic variation of the target gene have been developed. Two commonly used PCR-RFLP methods for DNA fingerprinting App strains are either using *aroA* gene or *apxIV* gene as target genes.² However, it is not known whether these target genes are encoding protective antigens or not.

During the past two decades many suspected virulence factors of App have been studied. Some known virulence genes have been identified, such as those involved in biosynthesis of capsule and lipopolysaccharide (LPS). A large number of genes that were not previously implicated in pathogenesis of App infection have also been identified.³ It has been reported that the virulence of each App serovar 7 strain is partly dependent on the type of the capsular polysaccharide (CP) it possesses.⁴ This is because CP is required for protection of App strains from the phagocytosis and complement-mediated killing of App in the host animal body. Also found was that App is resistant to complement-mediated killing, even in the presence of specific antibody. In contrast, capsule-deficient mutants were sensitive to the killing in normal serum.⁵ Variation in CP content may contribute to the difference in virulence among App field strains. Recently, it was also found that bacterial enzymes involved in anaerobic respiration also play a role in App persistence and virulence. Among them,

aspartase encoded by *aspA* gene of APP was found to be important for the survival of App in necrotic tissues and also required for long term colonization of intact respiratory epithelium.⁶ Ferric uptake regulatory protein (Fur), encoded by the *fur* gene of APP, has been shown to regulate virulence and virulence-associated factors.⁷

The aim of this study was to compare the serovar 7 App field strains using PCR assays to reveal the divergence or absence of some important App genes that had been predicted to encode virulence factors. These include genes encoding App's CP, aspartase, and ferric uptake regulatory protein. In this study, soluble protein profile, glycoprotein profile, and Western blot profile were also created for the comparison of App serovar 7 field strains.

Materials and methods

Detection of *cps* gene by PCR assay: The *cps*-specific primers (Table 1) were used to generate a 560-bp DNA amplicon. PCRs were performed in a final volume of 50 ul containing of 5 ul of DNA template, 5 ul of 10XPCR buffer, 1 ul of dNTP (2.5 mM each), 4.8 ul of each primer, 2.5 U of Taq DNA polymerase, 8 ul of magnesium chloride (25 mM), in 20.9 ul deionized water. The PCR mixtures were denatured at 94°C for 2 minutes, and 30 cycles at 94°C for one minute, 50°C for two minutes, and 72°C for two minutes, and then 72°C for one minute. The PCR products were run on a 2% agarose gel stained with ethidium bromide (4).

Detection of *aspA* and *fur* genes by PCR assay: PCRs were performed in a final volume of 50 ul containing 5 ul of DNA template, 5 ul of 10XPCR buffer, 1 ul of dNTP (2.5 mM each), 0.50 ul of each primer (40 uM each), 3 ul of magnesium chloride (25 mM), 0.2 ul of Taq (5 U/ul), and 27.8 ul of deionized water. The PCR mixtures were denatured at 92°C for 3 minutes, and 30 cycles at 94°C for one minute, 60°C for one minute, and 72°C for one minute, and then 72°C for five minutes.⁸

Electrophoresis, Western blotting and glycoprotein staining: Total soluble protein fractions were prepared

Table 1: Primers used for this study

Primer	Sequence
<i>aspX</i>	5'-TGGGCCGTA CT CAGTTACAA-3'
<i>aspY</i>	5'-GGGCCTGATGAAAGTAAACG-3'
<i>furX</i>	5'-GAACGTGTAAACCGTTGGTG-3'
<i>furY</i>	5'-GCCTGCAAACCTTCGGTAT-3'
<i>cps1U2</i>	5'-ATGGGTTACCTTTATTGAT-3'
<i>cps1L3</i>	5'-CCAATACATATCATAAAGAGCCG-3'

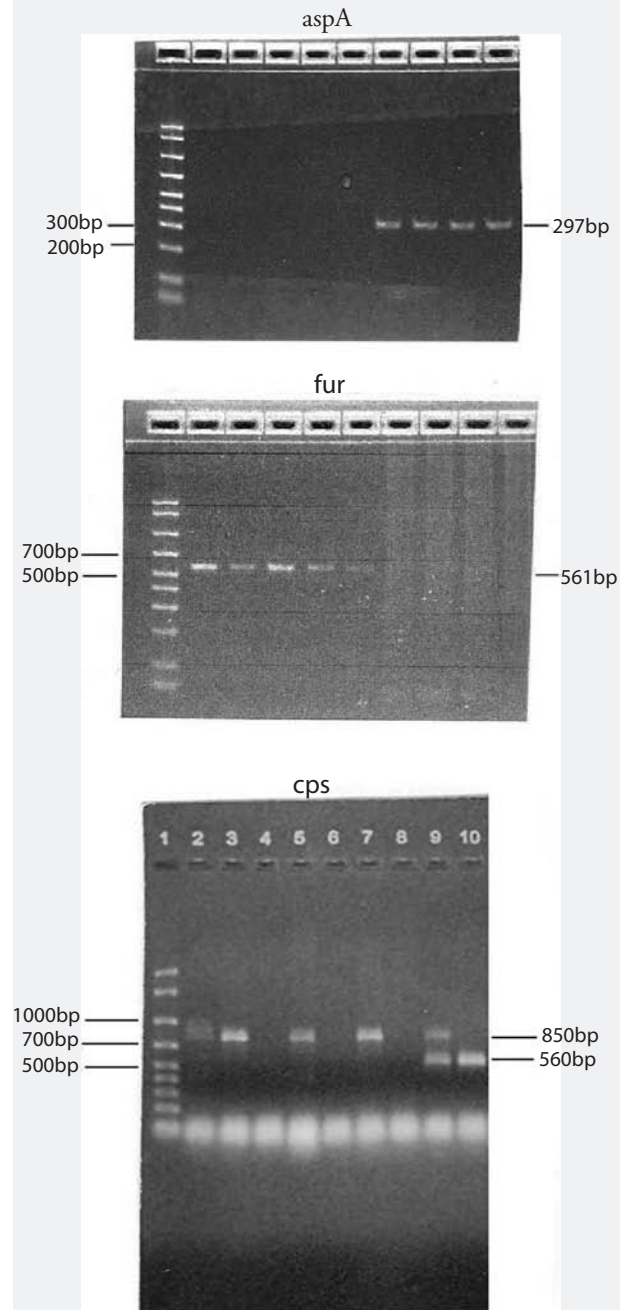
and analyzed by SDS-PAGE and Western blotting. In brief, one ml of the App culture was centrifuged and the cell pellet was reconstituted with 0.5 ml of a bacterial cell lysis/extraction reagent (CellLytic B, Sigma B7310) and stored at 4°C for an hour. The soluble protein fractions were obtained by centrifugation at 13,000 × G for 3 minutes. Electrophoresis and Western blotting were performed as described previously.⁹ The serovar 7 App field strains were studied for their antigenic heterogeneity using rabbit polyclonal hyperimmune sera. The glycoprotein staining was performed according to manufacturer's instruction using GelCode Glycoprotein Staining Kit (Pierce Biotechnology).

Determination of aspartase activity: The aspartase activity of each App serovar 7 strain was measured at 240 nm by determination of fumarate formation as described before.⁶ In brief, the whole cell lysate obtained from App colonies grown over night on Chocolate agar plate under anaerobic condition was used as the test material. The assay buffer contained 3 mM magnesium chloride, 0.1 M L- aspartate (Sigma), and 0.1 M Tris HCl (pH 9.0). The reaction was initiated by adding cell lysate to the assay buffer and the increase in absorbance at 240 nm was determined. Aspartase activity was expressed in units per mg of cell lysate, with 1 U being the amount that converts 1.0 nano mole of L-aspartate to fumarate per minute.

Results and discussion

Each of the App serovar 7 strains isolated from infected pigs can be characterized and evaluated using the above mentioned test methods to determine the presence or absence of some important virulence genes (*cps*, *fur*, *aspA*), its aspartase activity in the cell lysate, and its immunoblot pattern. In this study, it was found that antigenic and genetic diversity is present among the App serovar 7 field strains (Figure 1-4). Although several PCR-RFLP-based methods have been proposed for typing of App field isolates (2, 10), it is not known wheth-

Figure 1: PCR analyses of *A pleuropneumoniae* field strains for the presence of *aspA*, *fur*, and *cps* genes. Lanes 1: DNA size marker (50-2,000 bp); lanes 2 to 10: nine App serovar 7 field strains. The numbers to the right indicate the size of the PCR amplicon obtained.



er the genes that were typed really encode the protective antigens or not. The PCR assays used in this study can detect the presence of three most important virulence genes (Figure 1) and the aspartase activity test further characterizes the level of *aspA* gene expression under the anaerobic growth condition (Table 2). A recent report

Table 2: Aspartase activity in cell lysates of twelve App serovar 7 strains grown under anaerobic condition.

App serovar 7 strain	Aspartase activity (U/mg of cell lysate)
MVP09270	112
MVP09058	112
MVP08980	80
MVP08976	100
MVP08952	88
MVP08940	107
MVP08925	79
MVP08890	98
MVP08014	206
MVP07990	103
MVP07952	88
MVP06487	337

indicated that *aspA* but not *dmsA* is important for App's anaerobic growth and the aspartase may play a role in App virulence and persistence in necrotic lesions (6). The intra-serovar differentiation among App serovar 7 strains can be done by a comparison of the total protein profile, western blot profile, and glycoprotein profile of the cell lysate from each strain (Fig 2-5). The glycoproteins in prokaryotes have some known specific functions, such as maintenance of cell shape and protein stability, protection against proteolysis, and adherence of App to the target receptors on the host cells.¹¹ Fig 5 indicates that there exists difference in the glycoprotein profile among 12 different App serovar 7 strains.

It is known that aspartase play a major function in App virulence. It is because App's aspartase can convert aspartic acid to fumarate which can act as an electron acceptor for App's anaerobic respiration. In this study it

was found that the aspartase activity of 12 App strains is different and its range is from 79 U/mg to 337 U/mg. Further study to correlate the level of App's aspartase activity and its virulence in host animal is needed.

Antigenic heterogeneity among App serovar 7 field strains had been found in this study. And to examine the antigenic profile of each App field strain isolated from a pig herd is critical for the choice of autogenous vaccine strain. The PCR assays, immunoblotting, glycoprotein staining, and aspartase activity test used in this study will offer an alternative way to perform an intra-serovar differentiation and evaluation of App serovar 7 strains. This will not only offer an effective epidemiological analysis of porcine pleuropneumonia caused by App serovar 7 but also help swine practitioners choose the right vaccine strains for the control of this disease.

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Figure 2: A Colloidal-blue-stained SDS-PAGE profile of eight App serovar 7 strains. Lane 1: Precision Plus Protein Standards (BIO-RAD); lane 2 to 9: soluble proteins profile from each App serovar 7 strain.

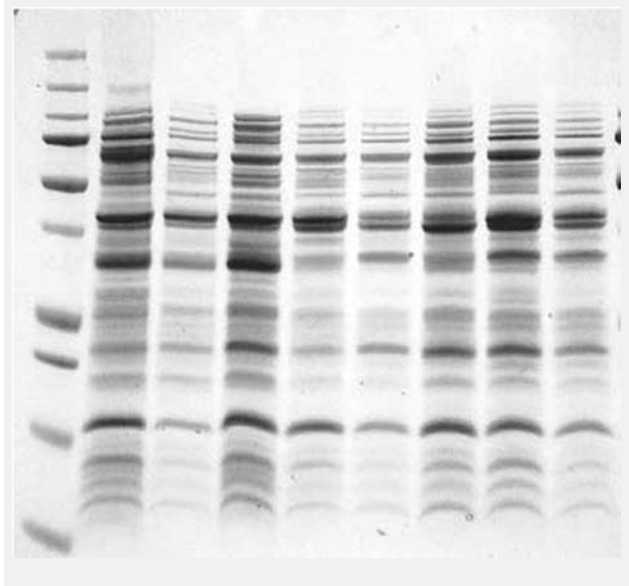


Figure 3: A dendrogram that was created based on the soluble protein profile of the eight App serovar 7 strains shown in fig. 2.

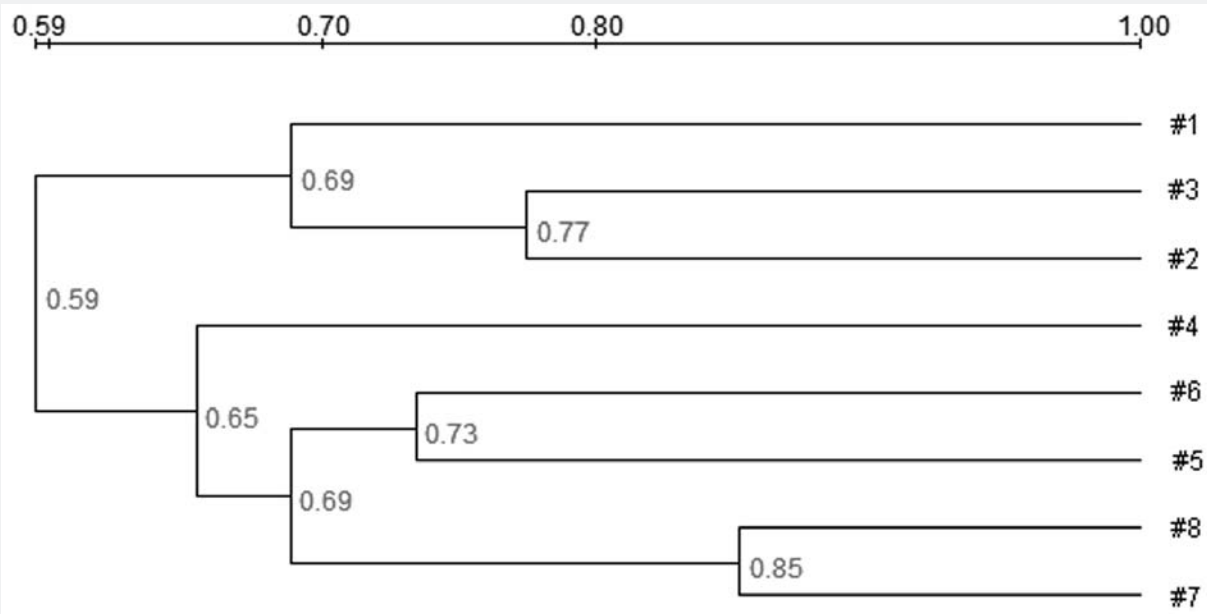


Figure 4: Western blot of ten App serovar 7 field strains with hyperimmune rabbit serum against App serovar 7. Lane 1: MagicMark XP Western Protein Standard (Invitrogen); lane 2 to 11: ten App serovar 7 strains.

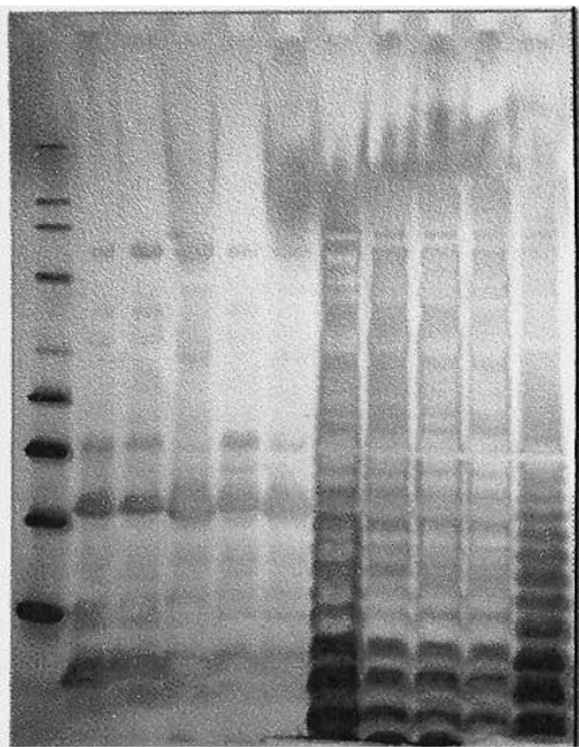
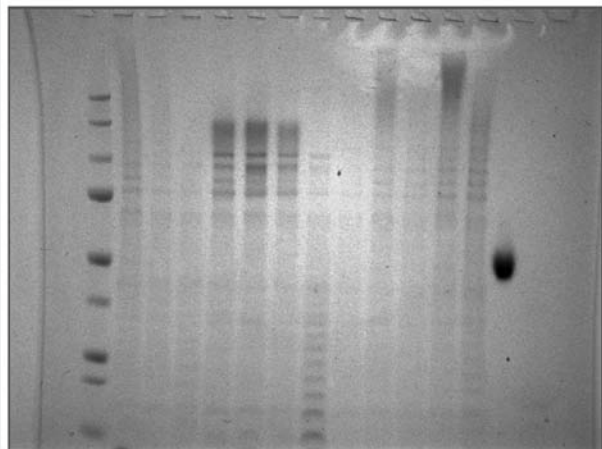


Figure 5: Detection of glycoproteins in the whole cell lysate of twelve App serovar 7 strains using SDS-PAGE and GelCode Glycoprotein staining kit. Lane 1: Precision Plus Protein standards (BIO-RAD); Lane 2 to 13: twelve App serovar 7 strains; Lane 14: 5 ug of horseradish peroxidase as positive control; Lane 15: 5 ug of soybean trypsin inhibitor as negative control.



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