

## Development of a Multiplex Polymerase Chain Reaction Method for *cps* Typing of *Actinobacillus pleuropneumoniae* Serovars 1, 2, 5, 7, and 15

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### Abstract

We developed a multiplex polymerase chain reaction (PCR) method using primers specific to the capsular polysaccharide biosynthesis genes (*cps*) to type *Actinobacillus pleuropneumoniae* serovars 1, 2, 5, 7, and 15. This multiplex PCR method may be useful for the typing of serovar 15, which is recently becoming more prevalent, as well as the most prevalent serovars 1, 2, 5, and 7 in Japan.

**Discipline:** Animal health

**Additional key words:** pig, pleuropneumonia, serotyping

### Introduction

*Actinobacillus pleuropneumoniae* is an etiologic agent of porcine pleuropneumonia that causes serious economic losses in the pig-rearing industry (Gottschalk 2012). Fifteen serovars of *A. pleuropneumoniae* have been identified to date, mainly based on the antigenic diversity of their capsular polysaccharides (Blackall et al. 2002, Perry et al. 1990). The prevalent local serovars vary among countries (Gottschalk 2012); in Japan, serovars 2 (59%), 1 (28%), 5 (8%), and 7 (3%) account for approximately 98% of the strains isolated from pigs (Asawa et al. 1995, Fukuyasu et al. 1991, 1996, Kume & Nakai 1988, Morioka et al. 2006, 2008, Suzuki et al. 1989, Yoshimura et al. 2002). However, the incidence of isolated cases of serovar 15, which is the most recently identified serovar (Blackall et al. 2002), has been recently increasing in Japan (Ito 2013, Morioka et al. 2008).

Because the virulence of strains differs depending on their serovars and differences in serovars affect the effectiveness of vaccines (Gottschalk 2012), serotyping is widely performed in veterinary diagnostic laboratories. However, cross-reactions are often observed among different serovars, for example, among serovars 1, 9, and 11 (Gottschalk 2012), serovars 3, 6, 8, and 15 (Gottschalk 2012), serovars 4 and 7 (Gottschalk 2012), and serovars 7 and 15 (Blackall et al. 2002, Koyama et al. 2007), prevent-

ing the accurate and rapid typing of field strains.

In the last decade, serovar-specific polymerase chain reaction (PCR) typing methods based on the sequence variation in genes involved in capsular polysaccharide biosynthesis (*cps*) have been developed for serovars 1-3, 5-8, 10, 12, and 15 (Angen et al. 2008, Bossé et al. 2014, Ito 2010, Jessing et al. 2003, Lo et al. 1998, Schuchert et al. 2004, Turni et al. 2014, Zhou et al. 2008). These PCR typing methods facilitate the reliable typing of *A. pleuropneumoniae* serovars without cross-reactions and mistyping. In the present study, we developed a multiplex PCR method for *cps* typing of *A. pleuropneumoniae* serovars 1, 2, 5, and 7, which are the most prevalent in Japan (Asawa et al. 1995, Fukuyasu et al. 1991, 1996, Kume & Nakai 1988, Morioka et al. 2006, 2008, Suzuki et al. 1989, Yoshimura et al. 2002), as well as serovar 15, isolated cases of which have been increasing (Ito 2013, Morioka et al. 2008).

### Materials and methods

We used the *A. pleuropneumoniae* serovar reference strains serovar 1, 4074; serovar 2, S1536; serovar 3, S1421; serovar 4, M62; serovar 5a, K17; serovar 6, Femø, serovar 7, WF83, serovar 8, 405; serovar 9, CVJ13261; serovar 10, D13039; serovar 11; 56153; serovar 12, 8329; serovar 13, N273; serovar 14, 4906; and serovar 15, HS143, to determine suitable conditions for multiplex PCR. Seventy-

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eight Japanese field strains of *A. pleuropneumoniae* serovar 1 (n=15), serovar 2 (n=15), serovar 5 (n=15), serovar 7 (n=13), serovar 8 (n=3), serovar 12 (n=3), and serovar 15 (n=12), as well as Canadian serovar 10 strains (n=2) were examined to assess the feasibility of using our new method. These strains were isolated from various areas and over several decades (from 1976 to 2011). The organisms were cultivated on TSA agar (Difco, Sparks, MD) supplemented with 5% defibrinated horse blood and 2% fresh yeast extract at 37°C.

Template DNAs of the serovar references and field strains were extracted with the High Pure PCR Template Preparation Kit and High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). Multiplex PCR was performed in a total volume of 50 µL containing 1 × buffer (Toyobo, Tokyo), 0.2 mM of each dNTP, 0.3 µM of each serovar- and species-specific primer (Table 1), 2.5 units of KOD FX Neo (Toyobo, Tokyo), and 10 ng of template DNA. The serovar 1, 2, 5, and 7- specific primers (Angen et al. 2008, Jessing et al. 2003, Lo et al. 1998) and species-specific primers (Gram & Ahrens 1998) used in this study were previously reported (Table 1). New primers

for serovar 15 were also designed based on the nucleotide sequence of the *cps15C* gene (Accession number: AB701753), which has been reported as specific to serovar 15 (Ito & Sueyoshi 2015). The amplification steps used were as follows: 94°C for 2 min (preheating); 35 cycles of 98°C for 10 sec (heat denature), 64°C for 30 sec (annealing), and 68°C for 1 min (extension); and a final step at 68°C for 5 min. Five µL of each reaction was analyzed by electrophoresis with a 2% agarose gel.

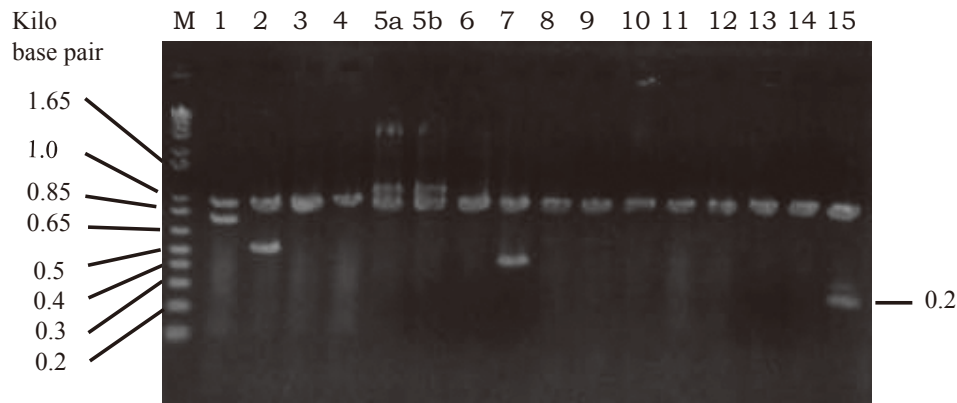
## Results

The reference strains of *A. pleuropneumoniae* serovars 1, 2, 5, 7, and 15 produced 0.75-, 0.5-, 1.1-, 0.4- and 0.2-kb PCR products, respectively (Fig. 1). In contrast, the reference strains of serovars 3, 4, 6, and 8-14 did not yield any serovar 1-, 2-, 5-, 7- or 15- specific PCR products (Fig. 1). All serovar reference strains produced 0.95-kb *A. pleuropneumoniae*-specific amplicons (Fig. 1).

The field strains of *A. pleuropneumoniae* serovars 1, 2, 5, 7, and 15 produced 0.75-, 0.5-, 1.1-, 0.4- and 0.2-kb PCR products, respectively (data not shown) as did the

**Table 1. Primers used for PCR amplification**

Primer name	Function	Sequence	Amplicon size (kilobase pairs)	DNA amplified	References
Ap1F	Forward primer for <i>cps</i> region of serovar 1	GGG CAA GCC TCT GCT CGT AA	0.75	serovar 1	Angen et al. 2008
Ap1R	Reverse primer for <i>cps</i> region of serovar 1	GAA AGA ACC AAG CTC CTG CAA T			Angen et al. 2008
Ap2F	Forward primer for <i>cps</i> region of serovar 2	ACT ATG GCA ATC AGT CGA TTC AT	0.5	serovar 2	Jessing et al. 2003
Ap2R	Reverse primer for <i>cps</i> region of serovar 2	CCT AAT CGG AAA CGC CAT TCT G			Jessing et al. 2003
Ap5A	Forward primer for <i>cps</i> region of serovar 5	TTT ATC ACT ATC ACC GTC CAC ACC T	1.1	serovar 5	Lo et al. 1998
Ap5B	Reverse primer for <i>cps</i> region of serovar 5	CAT TCG GGT CTT GTG GCT ACT AAA			Lo et al. 1998
Ap7F	Forward primer for <i>cps</i> region of serovar 7	GGT GAC TGG CGT ACG CCA AA	0.4	serovar 7	Angen et al. 2008
Ap7R	Reverse primer for <i>cps</i> region of serovar 7	GGG CTG CAG ACT GAC GTA A			Angen et al. 2008
Ap15F	Forward primer for <i>cps</i> region of serovar 15	GCA GAT TTG GGA TTC CGA TTT CGG	0.2	serovar 15	This study
Ap15R	Reverse primer for <i>cps</i> region of serovar 15	AGC AAC TTC AAT AAT CTG ACG ACG			This study
LPF	Forward primer for <i>omlA</i> gene	AAG GTT GAT ATG TCC GCA CC	0.95	serovars 1 to 15	Gram & Ahrens 1998
LPR	Reverse primer for <i>omlA</i> gene	CAC CGA TTA CGC CTT GCC A			Gram & Ahrens 1998



**Fig. 1. Multiplex PCR results with serovar reference strains. The number above each lane indicates the serovar. M: 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA).**

serovar reference strains. The field strains of serovars 8, 10, and 12 yielded faint PCR products with a band size slightly exceeding that amplified from the serovar 2 strain, although these field strains did not yield any serovar 1-, 2-, 5-, 7- or 15-specific PCR products. However, each faint DNA band could be easily discriminated from the intense bands from serovars 1, 2, 5, 7, and 15. All field strains produced 0.95-kb species-specific amplicons.

## Discussion

In the present study, we focused on developing a multiplex PCR method for *cps* typing of *A. pleuropneumoniae* serovars 1, 2, 5, and 7 because they are the most predominant serovars in Japan, accounting for approx. 98%, as well as the newly identified serovar 15, since its incidence in the form of isolated cases has recently been increasing. This multiplex PCR method may be useful, not only in Japan, but also worldwide, since serovars 1, 5, and 7 in North America (Gottschalk 2012), serovar 2 in most of Europe (Gottschalk 2012), and serovar 15 in Australia (Blackall et al. 2002) are the most dominant serovars causing most outbreaks in these regions. In conclusion, the present multiplex PCR method for serovars 1, 2, 5, 7, and 15 may be used as a valuable tool complementing the serotyping of *A. pleuropneumoniae* field strains for diagnoses and epidemiological studies.

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