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Evaluation of a Multiplex PCR Test for Simultaneous Identification and Serotyping of *Actinobacillus pleuropneumoniae* Serotypes 2, 5, and 6

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Serotype-specific DNA regions involved in the biosynthesis of capsular polysaccharides (*cps* region) were used to develop a multiplex PCR test for the simultaneous species identification and serotyping of *Actinobacillus pleuropneumoniae* serotypes 2, 5, and 6. Primers specific for serotypes 2, 5, and 6 were combined with the already existing species-specific primers used in a PCR test based on the *omlA* gene. The PCR test was evaluated with serotype reference strains of *A. pleuropneumoniae* as well as 182 Danish field isolates previously serotyped by latex agglutination or immunodiffusion. For all serologically typeable strains, a complete correspondence was found between the results obtained by the multiplex PCR test and the results obtained by the traditional serotyping methods. Six of eight serologically nontypeable strains could be allocated to a serotype on the basis of the multiplex PCR results. The species specificity of the assay was evaluated with a collection of 93 strains representing 29 different species within the family *Pasteurellaceae*, as well as species normally found in the respiratory tracts of swine. All of these strains were negative by the multiplex PCR test, including 50 field isolates of the phylogenetically closely related species *Actinobacillus lignieresii*. When the multiplex PCR test was used to test Danish field strains, it was able to identify the serotypes of approximately 94% of all strains isolated from swine with clinical disease. More than 90% of the isolates that cross-reacted by the latex agglutination test were of serotype 2, 5, or 6. Determination of the serotype by PCR represents a convenient and specific method for the serotyping of *A. pleuropneumoniae* in diagnostic laboratories.

Actinobacillus pleuropneumoniae is an encapsulated respiratory pathogen of swine and the causative agent of porcine pleuropneumonia (28). The disease occurs worldwide and has resulted in large economic losses to the swine industry. At present, 15 different serotypes and 2 biotypes have been described (1, 6, 20, 23). The serotype specificity is predominately due to structural differences in the capsular polysaccharides (22). The presence and prevalence of serotypes vary among countries. In North America, serotypes 1 and 5 are the most commonly isolated, whereas serotypes 2 and 9 are isolated in many European countries (14, 27). In Denmark, serotypes 2 (63%), 5 (5%), and 6 (26%) account for approximately 94% of the strains isolated from swine with clinical disease. Virulence studies indicate considerable differences in virulence between serotypes (5, 11, 25).

A number of serological assays have been developed for the serotyping of *A. pleuropneumoniae*, but cross-reactions between serotypes are often seen by rapid serological assays such as slide agglutination tests. Definitive typing of such isolates has been achieved by using more time-consuming procedures, for example, immunodiffusion and indirect hemagglutination. Serological cross-reactivity between serotypes 1 and 9, serotypes 3, 6, and 8, and serotypes 4 and 7 has been reported (16, 17, 18). These cross-reactions are most likely due to the presence of similar antigens in the lipopolysaccharides (22). One way to avoid this serological cross-reactivity is to use DNA-based assays for the identification of *A. pleuropneumoniae*. During the last decade PCR has become a powerful DNA-based tool for the identification of microbes (24). A number of species-specific PCR tests have been developed for identification of *A. pleuropneumoniae* (8, 19, 26, 29), and a serotype-specific multiplex PCR assay has been designed for the simultaneous identification and serotyping of *A. pleuropneumoniae* serotype 5 (13).

The aim of the present investigation was to develop a PCR assay for the simultaneous species identification and serotyping of *A. pleuropneumoniae* serotypes 2, 5, and 6, which will be of great practical importance in diagnostic laboratories where these are the most prevalent serotypes. The PCR test is based on amplification of serotype-specific DNA regions involved in the biosynthesis of the capsular polysaccharides (*cps* genes). Oligonucleotide primers specific for the *cps* regions of serotypes 2, 5, and 6 were combined with primers previously used for the species-specific PCR amplification of the *omlA* gene (8). The specificity and sensitivity of the multiplex PCR test were examined, and the results of the multiplex PCR were compared with those obtained by traditional serological typing methods.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used for this study are listed in Tables 1 and 2. In addition to reference strains representing serotypes 1 to 15, a total of 182 Danish field isolates of *A. pleuropneumoniae* from pig lungs representing isolates of serotypes 1 ($n = 5$), 2 ($n = 46$), 5 ($n = 51$), 6 ($n = 56$), 7 ($n = 5$), 8 ($n = 5$), and 12 ($n = 6$) and nontypeable isolates ($n = 8$) were included in the study. In addition, 93 strains representing 29 genetically related species, as well as other species normally found in the respiratory tracts of pigs,

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TABLE 1. A. pleuropneumoniae reference strains and field isolates used for evaluation of the PCR test

A. pleuropneumoniae species	Strain designation	No. of strains	No. of strains positive by PCR with:	
			A. pleuropneumoniae-specific amplicon (approx 950 bp)	Serotype-specific amplicon
Reference isolates				
Serotype 1	Shope 4074^T	1	1	0
Serotype 2	S 1536	1	1	1 (500)[a]
Serotype 3	S 1421	1	1	0
Serotype 4	M62	1	1	0
Serotype 5A	K17	1	1	1 (1,100)
Serotype 5B	L20	1	1	1 (1,100)
Serotype 6	Femϕ	1	1	1 (720)
Serotype 7	WF83	1	1	0
Serotype 8	405	1	1	0
Serotype 9	CVJ 13261	1	1	0
Serotype 10	D 13039	1	1	0
Serotype 11	56153	1	1	0
Serotype 12	8329	1	1	0
Serotype 13 (biovar 2)	N-273	1	1	0
Serotype 14 (biovar 2)	3906	1	1	0
Serotype 15	HS143	1	1	0
Danish field isolates				
Serotype 1		5(5)[b]	5	0
Serotype 2		46(23)	46	46 (500)
Serotype 5		51(36)	51	51 (1,100)
Serotype 6		56(46)	56	56 (720)
Serotype 7		5(4)	5	0
Serotype 8		5(2)	5	0
Serotype 12		6(2)	6	0
Nontypeable strains		8	8	5 (500) 1 (720) (−)

[a] The values in parentheses indicate the approximate amplicon size (in base pairs).
[b] The values in parentheses indicate the number of strains cross-reacting by latex agglutination.

were used to evaluate the species specificity of the multiplex PCR test. This included 50 field isolates of the phylogenetically closely related species *Actinobacillus lignieresii.* All V-factor-dependent strains were grown on PPLO agar (Difco, Detroit Mich.), whereas all other strains tested were grown on Columbia agar supplemented with 5% bovine blood. All strains were incubated at 37°C overnight in atmospheric air.

Serotyping of *A. pleuropneumoniae*. All the Danish field isolates of *A. pleuropneumoniae* were serotyped by a latex agglutination test (7). Each isolate was tested with latex particles coated with polyclonal antibodies produced against whole cells of reference strains of *A. pleuropneumoniae* representing serotypes 1 through 15 (Table 1). If the latex agglutination test showed a cross-reaction between different serotypes, the isolates were also tested by immunodiffusion or indirect hemagglutination to determine the serotype (21).

Oligonucleotide primers for PCR. The sequences of the oligonucleotide primers used in this study are listed in Table 3. Four different pairs of primers were used in the multiplex PCR test. Serotype-specific primers were designed from the *cps* genes of serotypes 2, 5, and 6. The primers specific for serotype 2 and serotype 6 were designed in this study, while the primers specific for serotype 5 were designed in an earlier study (13). These three serotype-specific primers were combined with primers used in a previously published species-specific PCR test based on amplification of the *omlA* gene (8). The DNA primers designed in this study were selected by using the DNASIS sequence analysis program (Hitachi Software Engineering Co., Ltd.).

Preparation of samples for PCR. Lysates of pure cultures were prepared for all strains used in this study. A loopful of an overnight culture was taken from the surface of an agar plate and suspended in 200 μl of sterile water. The suspension was boiled for 10 min, and the cells were spun down at 13,000 × *g* for 2 min. A total of 180 μl of the supernatant was kept for analysis. The lysates were used undiluted as the DNA template in the PCR. The lysates were stored at −20°C until use.

PCR amplification. PCR was performed in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCl; 0.005% Tween 20; 0.005% Nonidet P-40 detergent; 200 μM (each) dATP, dCTP, dGTP, and dTTP; and 1 U of *Taq* polymerase (Perkin-Elmer). The primers (Table 3) were added at the following final concentrations: 0.4 μM for HPF, HPR, SGJ5, and SGJ14; 2.0 μM for Ap5A and Ap5B; and 0.1 μM for Ap2F and Ap2R. One microliter of undiluted template DNA was added to each reaction mixture. Finally, mineral oil was added to prevent evaporation. The PCRs were performed in a Biometra Trio thermocycler by using 0.5-ml tubes. DNA was amplified for 33 cycles by using the following settings: denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min 20 s. Twelve microliters of each reaction mixture was analyzed by electrophoresis in a 2% agarose gel. The PCR products were stained with ethidium bromide (10 μg/ml) and visualized under UV light.

RESULTS

Latex agglutination of *A. pleuropneumoniae* strains. Of the 182 Danish field isolates of *A. pleuropneumoniae*, only 56 strains could be serotyped by latex agglutination test with polyclonal antibodies to whole cells. The remaining 126 isolates showed cross-reactions between two or more serotypes by the latex agglutination test. The serotypes of these 126 isolates were identified by immunodiffusion or indirect hemagglutination. Eight *A. pleuropneumoniae* strains could not be allocated to a serotype by any of these methods (Table 1).

Optimization of multiplex PCR test. Two pairs of oligonucleotide primers were designed to amplify a part of the serotype-specific *cps* region from serotype 2 and 6 isolates, respectively. Primers Ap2F and Ap2R were designed to produce an approximately 500-bp PCR fragment from serotype 2 isolates,

TABLE 2. Collection of strains not giving rise to any amplicons in the multiplex PCR test

Species	Strain(s) designation[a]	No. of strains
Actinobacillus actinomycetemcomitans	CCUG 13227^T	1
Actinobacillus capsulatus	NCTC 11408^T, P1364	2
Actinobacillus equuli	NCTC 8529^T, P1284	2
Actinobacillus hominis	NCTC 11529^T, P1336	2
Actinobacillus indolicus	46KC2^T, 39Cl	2
Actinobacillus lignieresii	ATCC 49236^T, 50 field isolates	51
Actinobacillus minor	NM 305^T	1
Actinobacillus porcinus	NM 319^T, 18765-5(T1), 18870-2(T5), 18870-2(T7), 595	5
Actinobacillus rossii	ATCC 27072^T	1
Actinobacillus suis	CAPM 5586^T, P1143	2
Actinobacillus urea	NCTC 10219^T, P1144	2
Haemophilus parainfluenzae	CCUG 12836^T	1
Haemophilus paraphrophilus	3718	1
Haemophilus parasuis	NCTC 4557^T	1
Haemophilus segnis	CCUG 10787^T	1
Mannheimia haemolytica	NCTC 9380^T	1
Mannheimia varigena	CCUG 16500, CAPM 5786, 3942/87	3
Pasteurella aerogenes	ATCC 27883^T	1
Pasteurella avium biovar 2	Strain 5	1
Pasteurella canis	CCUG 12400^T	1
Pasteurella canis biovar 2	Strain 25	1
Pasteurella langaa	CCUG 15566^T	1
Pasteurella mairii	CCUG 27189^T	1
Pasteurella multocida	NCTC 10320	1
Pasteurella multocida subsp. *septica*	HIM 746-6	1
Pasteurella stomatis	CCUG 17979^T	1
Pasteurella sp. strain *B*	CCUG 19794	1
Bordetella bronchiseptica	1317	1
Streptococcus suis	735, 8074, 24636/74	3

[a] ATCC, American Type Culture Collection, Manassas, Va.; CAPM, Collection of Animal Pathogenic Microorganisms, Brno, Czech Republic; CCUG, Culture Collection University of Gothenburg, Gothenburg, Sweden; NCTC, National Collection of Type Cultures, London, United Kingdom.

whereas primers SGJ14 and SGJ5 were designed to produce an approximately 720-bp PCR fragment from serotype 6 isolates. The Ap5A-Ap5B primer pair amplifies an approximately 1,100-bp portion of the serotype-specific *cps* region from serotype 5 isolates (13). These three primer pairs were combined with the primers used in an existing species-specific *A. pleuropneumoniae* PCR test that produce an approximately 950-bp amplicon from isolates of all *A. pleuropneumoniae* serotypes (8).

For optimization of the multiplex PCR test, serotype reference strains of *A. pleuropneumoniae* serotypes 1 through 15 were used. The annealing temperature and the reaction buffer were the same as those used for the species-specific test based on amplification of *omlA* (8). The magnesium concentration was varied from 1 to 2.5 mM. With low magnesium concentrations some of the expected PCR fragments were faint or nondetectable, while with high magnesium concentrations nonspecific PCR products of various sizes were amplified. From these results the optimum magnesium concentration for the assay was determined to be 1.5 mM. The concentrations of all primers were set from the beginning to be 0.4 μM, which resulted in differences in the intensities of the amplified DNA fragments. Initially, the intensity of the serotype 2-specific amplicon was much more intense than those of the other PCR products produced. The intensities of the amplicons became almost identical when the concentration of the serotype 2 primers was lowered to 0.1 μM and the concentration of the serotype 5 primers was increased to 2.0 μM.

Serotype specificity of multiplex PCR test. The serotype specificity of the multiplex PCR was determined by applying the test to a collection of 198 *A. pleuropneumoniae* isolates, including the serotype reference strains (Table 1). The species-specific fragment of approximately 950 bp was amplified from all *A. pleuropneumoniae* strains tested. An additional serotype-specific fragment was amplified from strains belonging to serotypes 2, 5, and 6 (Fig. 1). All Danish field isolates of *A. pleuropneumoniae* typed as serotype 2, 5, or 6 by one of the

TABLE 3. Primers used for PCR amplification

Name	Function	Sequence (5′ → 3′)	Approx size of amplicon (bp)	DNA amplified
HPF[a]	Forward primer for *omlA* gene	AAG GTT GAT ATG TCC GCA CC	950	*A. pleuropneumoniae* serotypes 1 to 15
HPR[a]	Reverse primer for *omlA* gene	CAC CGA TTA CGC CTT GCC A		
Ap2F	Forward primer for *cps* region of serotype 2	ACT ATG GCA ATC AGT CGA TTC AT	500	*A. pleuropneumoniae* serotype 2
Ap2R	Reverse primer for *cps* region of serotype 2	CCT AAT CGG AAA CGC CAT TCT G		
Ap5A[b]	Forward primer for *cps* region of serotype 5	TTT ATC ACT ATC ACC GTC CAC ACC T	1,100	*A. pleuropneumoniae* serotype 5
Ap5B[b]	Reverse primer for *cps* region of serotype 5	CAT TCG GGT CTT GTG GCT ACT AA		
SGJ14	Forward primer for *cps* region of serotype 6	AAC CAC TCA CTT TCC ACA TTA G	720	*A. pleuropneumoniae* serotype 6
SGJ5	Reverse primer for *cps* region of serotype 6	AAT CGG AAG GTT TTG GTC TCG TG		

[a] The primers were designed in an earlier study by Gram and Ahrens (8).
[b] The primers were designed in an earlier study by Lo et al. (13).

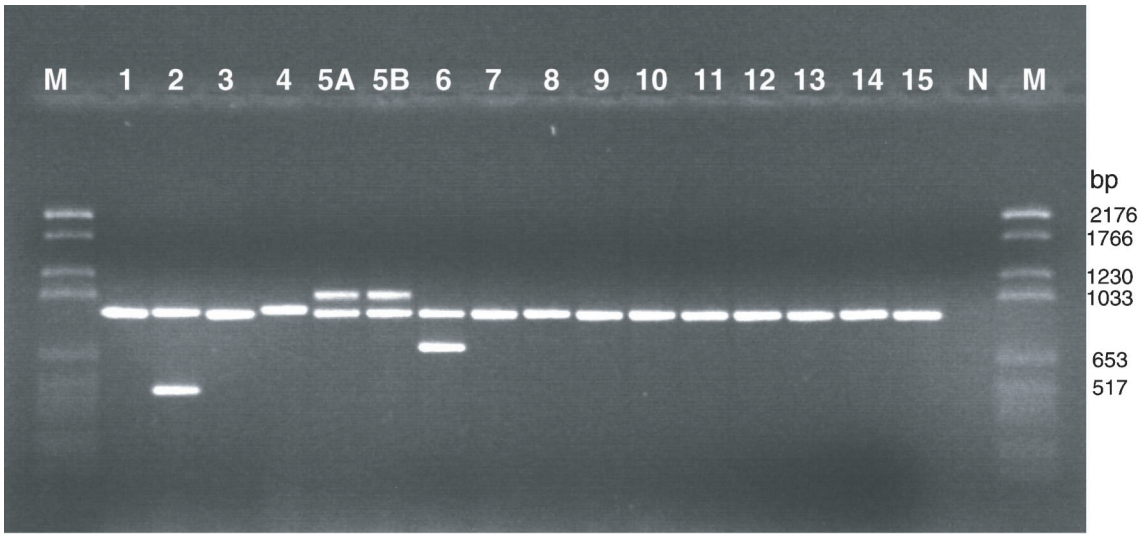

FIG. 1. Agarose gel electrophoresis of DNA fragments generated by multiplex PCR with the reference *A. pleuropneumoniae* strains of serotypes 1 to 15 (as indicated above the lanes) (Table 1). All strains gave rise to a species-specific band of approximately 950 bp, in addition to serotype-specific bands: approximately 500 bp (serotype 2), approximately 1,100 bp (serotypes 5A and 5B), and approximately 720 bp (serotype 6). Lane N, nontemplate control; lanes M, DNA molecular weight marker VI (Boehringer Mannheim).

traditional serotyping methods were allocated to the same serotype by the multiplex PCR test. Eight strains of *A. pleuropneumoniae* which could not be serotyped by latex agglutination, immunodiffusion, or indirect hemagglutination were tested. Five of these strains gave rise to an amplicon of the same size as that specific for serotype 2 (approximately 500 bp), and one of the strains gave rise to an amplicon of the same size as that specific for serotype 6 (approximately 720 bp). Two of the nontypeable strains gave rise only to the *A. pleuropneumoniae* species-specific fragment of approximately 950 bp, indicating that they did not belong to serotype 2, 5, or 6. Nonspecific PCR products were not observed from any of the strains when the optimized conditions for the multiplex PCR assay were used.

Species specificity of multiplex PCR test. The species specificity of the PCR assay was evaluated with a collection of 93 strains representing 29 different species within the family *Pasteurellaceae* as well as other species normally found in the upper respiratory tracts of swine (Table 2). The collection also included 50 field isolates of the phylogenetically closely related species *A. lignieresii*. The species-specific primers did not amplify the *A. pleuropneumoniae* species-specific amplicon of approximately 950 bp, and the serotype-specific primers did not amplify any product from any of the 93 heterologous strains tested in this study. Nonspecific PCR products were not observed from any of the related species tested when the optimized conditions for the multiplex PCR assay were used.

DISCUSSION

This is the first report of a PCR assay that simultaneously identifies *A. pleuropneumoniae* and three different serotypes. Cross-reactions between serotypes often occur when traditional serological assays are used for the serotyping of *A. pleuropneumoniae*. One way to avoid this serological cross-reactivity is to use DNA-based methods for serotyping, such as PCR, which does not involve antigens or antibodies. The present study shows that the use of multiplex PCR can be of great practical importance in the serotyping of *A. pleuropneumoniae*. A multiplex PCR test for the simultaneous detection of both the species *A. pleuropneumoniae* and serotypes 2, 5, and 6 was evaluated. The assay uses the advantage of multiplex PCR, in which more than one set of primers leads to the amplification of DNA from two or more target sequences in the same reaction under a single set of reaction conditions (15).

The design of the multiplex PCR test was determined by the distribution of *A. pleuropneumoniae* serotypes in Denmark. Almost 94% of the field strains isolated belong to serotype 2, 5, or 6. In Denmark, serotype 6 has been regarded as less virulent than serotype 2 and 5 in specific-pathogen-free animals. A quick and reliable means for the serotype designation of an isolate has therefore had a high priority in diagnostic bacteriology. Approximately 10% of the field isolates received at the Danish Veterinary Institute cross-react in the routinely performed latex agglutination tests, making it necessary to use more time-consuming serological serotyping procedures, such as immunodiffusion or indirect hemagglutination. More than 90% of these cross-reacting isolates represent serotype 2, 5, or 6. After optimization of the multiplex PCR assay, nonspecific amplicons were not observed from any of the isolates tested. All 182 Danish field isolates tested by the assay were identified as *A. pleuropneumoniae*; and all isolates identified as serotypes 2, 5, and 6 by serological assays were also identified as serotype 2, 5, and 6, respectively, by the multiplex PCR test. Furthermore, the multiplex PCR test was able to allocate six of the eight nonserotypeable isolates of *A. pleuropneumoniae* to either serotype 2 or serotype 6. These results show that the multiplex PCR test is a highly specific method for the identification and serotyping of *A. pleuropneumoniae* isolates and will be of great practical importance in the diagnostic laboratory.

Until now, most of the PCR assays reported for *A. pleuropneumoniae* have been used either for species identification or

for subtyping of an isolate and do not simultaneously identify the species and the serotype. Various PCR tests have been developed for species identification of *A. pleuropneumoniae* (2, 8, 19, 26, 29). A common feature of these PCR tests is that the primers used for amplification are intended to be specific for *A. pleuropneumoniae* and not for any of the *A. pleuropneumoniae* serotypes. These assays are used only for identification of the organism, and therefore, other methods are still needed for serotyping. Furthermore, in these assays primers specific for the target gene(s) often amplify DNA from closely related species, such as *Actinobacillus equuli* and *A. lignieresii* (2, 19, 29). In the present multiplex PCR test, the species-specific amplicon was observed only when strains of *A. pleuropneumoniae* were tested and not when other species were tested (Table 2). Other PCR-based assays have been designed for the subtyping of *A. pleuropneumoniae*. However, most of the PCR-based typing methods do not use primers for genes directly involved in capsule production, which results in a subtype that at best is associated with the serotype. Hennessy et al. (10) described the use of arbitrarily primed PCR for serotyping. This method is able to differentiate between serotypes 1 through 12 by using a combination of two tests, although some of the serotypes can be distinguished only by minor differences in band migration. Gram et al. (9) developed an *A. pleuropneumoniae*-specific PCR typing system based on the *apx* and *omlA* genes. This PCR typing system could discriminate the majority of *A. pleuropneumoniae* serotypes of biotype 1 except serotypes 1, 9, and 11 and serotypes 2 and 8. Furthermore, the PCR typing system failed to discriminate between serotype 6 isolates and field isolates of serotype 8.

A more optimal design for DNA-based serotyping would be an assay with genes that are unique to the different serotypes, which can make the test independent of contaminating DNA. Lo et al. (13) were the first to describe the use of a multiplex PCR to amplify conserved and serotype-specific DNA regions involved in encapsulation to simultaneously detect both *A. pleuropneumoniae* and serotype 5 isolates. The species-specific PCR included in that assay failed to amplify a DNA fragment from *A. pleuropneumoniae* serotype 4. Furthermore, the species specificity of the PCR test was evaluated with only a few species; the closely related species *A. lignieresii* was not included in that study.

The present multiplex PCR test did not amplify DNA fragments from any of the 29 different species related to *A. pleuropneumoniae* or species normally found in the respiratory tracts of swine (Table 2). These results indicate a 100% species specificity of the multiplex PCR assay. Furthermore, DNA from 50 field isolates of the closely related species *A. lignieresii* did not provide a template for the multiplex PCR. *A. lignieresii* is the species that is the most phylogenetically related to *A. pleuropneumoniae*, as determined by comparison of 16S rRNA sequences, and serological cross-reactions between the two species have been observed (3, 4, 12). These serological cross-reactions have been reported for serotypes 3, 4, and 7 and consequently do not involve any of the serotypes for which the present multiplex PCR test is specific. Furthermore, the nature of these serological cross-reactions might rely on lipopolysaccharide or protein antigens. Future sequencing of the *cps* genes from more *A. pleuropneumoniae* serotypes might help to elucidate these cross-reactions.

In conclusion, the results obtained in this study indicate that the multiplex PCR test is a sensitive, specific, and highly effective diagnostic tool for the simultaneous identification and serotyping of *A. pleuropneumoniae* serotypes 2, 5, and 6. Moreover, problems with serological cross-reactions can be avoided in diagnostic laboratories by using multiplex PCR. The results also confirm that the genes involved in the biosynthesis of the capsular polysaccharides contain serotype-specific regions in *A. pleuropneumoniae* serotypes 2, 5, and 6. A future aim is to develop multiplex PCR tests based on the serotype-specific *cps* region that can allocate all isolates of *A. pleuropneumoniae* to a serotype.

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