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Multiplex PCR That Can Distinguish between Immunologically Cross-Reactive Serovar 3, 6, and 8 *Actinobacillus pleuropneumoniae* Strains▽

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We describe a highly sensitive and specific multiplex PCR, based on capsular loci and the species specific *apxIV* gene, that unequivocally differentiates serovar 3, 6, and 8 *Actinobacillus pleuropneumoniae* strains that are cross-reactive in conventional immunological tests.

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a disease that is responsible for substantial mortality, morbidity, and economic losses worldwide (48). The ability to discriminate between strains of bacterial pathogens, such as *A. pleuropneumoniae*, is advantageous in better understanding disease transmission, tracking virulent or antibiotic-resistant strains, and probing the nature of evolutionary biology (46). In the case of *A. pleuropneumoniae*, a number of different techniques have been used to discriminate between strains, including multilocus enzyme electrophoresis (14, 30, 31), HindIII ribotyping (11, 12, 16), PCR-restriction endonuclease analysis (16, 50) or PCR-restriction fragment length polymorphism (PCR-RFLP) (6, 7), ApxI-III toxin typing alone (2, 10) or in combination with outer membrane lipoprotein A (*omlA*)-typing (13), and ApxI-IV toxin typing (39, 47). However, the gold standard method remains serotyping, to which all of the newer techniques are compared, but with which there is a correlation to differing extents. Conventional immunological serotyping of isolates involves the reactivity of, typically, rabbit serum to surface polysaccharides, i.e., capsule or lipopolysaccharide (9). Fifteen serovars have been described (3, 18, 19, 32, 33, 35, 37, 38, 42), although serovar 5 is subdivided into 5a and 5b (34). There are a variety of serotyping methods available, including complement fixation, indirect hemagglutination, enzyme-linked immunosorbent assay, agglutination and coagulation, latex agglutination, indirect fluorescent antibody labeling, immunodiffusion, and ring precipitation (9, 24). Serotyping has its limitations: there is little standardization between the various methods, and there can be considerable interbatch serum variation and cross-reactivity between different serovars, for example, between serovars 1 and 9 (27), serovars 4 and 7 (28), and serovars 3, 6, and 8 (29). In countries where any of the cross-reactive serovars are highly prevalent, strain differentiation can be highly problematic (9). This is particularly acute in the United Kingdom, where serovars 3, 6, and 8 predominate (1, 26). To overcome such limitations, phenotypic and genotypic methods have been developed to enable precise serotyping. Phenotypic methods include the development of monoclonal antibodies (4, 21, 40, 41), and genotypic methods include those based on the PCR amplification of capsular biosynthesis or export genes. PCRs for serovar 2 (15) and serovar 5 (25) strains and multiplex PCRs for the simultaneous identification of serovars 2, 5, and 6 (17) and serovars 1, 2, and 8 (45) have been published. Although serovar 6- and serovar 8-specific primers have been described as part of other multiplex PCRs (17, 45), to date, there has not been a description of a multiplex PCR that can discriminate between the cross-reactive serovar 3, 6, and 8 strains. In our previous study we described a serovar 3-specific *apxIV* multiplex PCR (53). The *apxIV* gene is specific to *A. pleuropneumoniae* and can be used to verify test strains to the species level (43). In the context of a multiplex PCR, the use of *apxIV* also allows evaluation of the integrity of genomic DNA (53). Unfortunately, despite exhaustive variations in experimental PCR parameters (buffer conditions, magnesium salt concentrations, annealing temperatures, primer concentrations, etc.), we were unable to combine our serovar 3- and *apxIV*-specific primers with those previously described for serovars 6 and 8 (17, 45). Here we describe a new set of serovar 3- and serovar 8-specific primers that can be multiplexed with those described for serovar 6 (17) and *apxIV* (53) to enable a highly sensitive and specific test that can discriminate between the problematic cross-reactive serovar 3, 6, and 8 strains in a single PCR.

In our previous study (53), to obtain the serovar 3-specific sequence, we exploited the conserved nature of gene loci involved in capsule expression that occurs in many gram-negative bacteria including *A. pleuropneumoniae* (51, 52). An oligonucleotide, AP5C, that annealed to a conserved consensus sequence in the capsular export region of *A. pleuropneumoniae* (45), combined with the arbitrary primer ARB6, resulted in a

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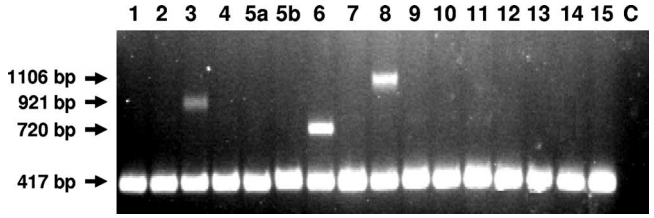

FIG. 1. *A. pleuropneumoniae apxIV* (417 bp), serovar 3-specific (921 bp), serovar 6-specific (718 bp), and serovar 8-specific (1,106 bp) amplicons. Lane numbers correspond to the serovar of the *A. pleuropneumoniae* reference strains. C, no DNA control.

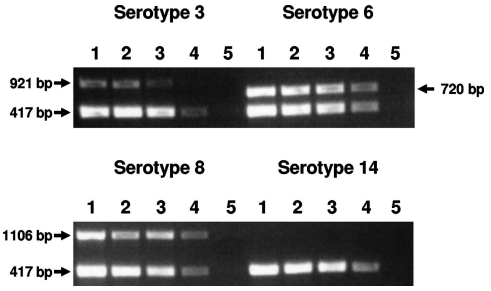

FIG. 2. *A. pleuropneumoniae apxIV* (417 bp) and serovar 3-specific (921 bp), serovar 6-specific (718 bp), or serovar 8-specific (1,106 bp) amplicons resulting from a 10-fold dilution series of starting genomic DNA template of the reference strains as follows. Lanes: 1, 21.4 ng; 2, 2.14 ng; 3, 214 pg; 4, 21.4 pg; 5, 2.14 pg. No amplicons were observed when genomic DNA was omitted (data not shown).

4-kb PCR amplicon that, after DNA sequencing of 2,732 bp, was used to identify two serovar 3-specific primers. In the present study, the 2,732 bp of DNA sequence available (GenBank accession number EU090171) was reanalyzed, and two further potential serovar 3-specific primers, AP3NR (5′-AAC AAA TAA AGT TGC TCG AAA GTA-3′) and AP3NF (5′-TTT GCG CTG TAG TGC TCC AAT-3′), were identified. In initial experiments, AP3NR and AP3NF were multiplexed with the primers APXIVA1 (5′-TTA TCC GAA CTT TGG TTT AGC C-3′) and APXIVA3 (5′-CAT ATT TGA TAA AAC CAT CCG TC-3′) specific for *apxIV* (53), the primers AP6F (5′-AAC CAC TCA CTT TCC ACA TTA G-3′) and AP6R (5′-AAT CGG AAG GTT TTG GTC TCG TG-3′) specific for serovar 6 used by Jessing et al. (17), and the primers AP8NR (5′-GAT TAA ACT GGT CCG TCG AAA TG-3′) and AP8NF (5′-TTA GTT GCG CAA ACG GCT TTT GAA-3′) specific for serovar 8, which were designed from the available sequence of the *cps8ABC* genes (accession number AY356527). The serovar 8 primers described by Shuchert et al. (45) were not used since these primers result in a PCR band of 970 bp, which is similar in size to that for serovar 3 (918 bp). The PCR conditions used were as follows: the optimal reaction mix (total volume 20 μl) contained 0.5 U of HotStarTaq DNA polymerase (Qiagen), 1× Qiagen PCR buffer, 2.0 mM magnesium chloride, 200 μM concentrations of each deoxynucleoside triphosphate, 0.5 μM concentrations of the primers AP3NF and AP3NR, 0.125 μM concentrations of the primers AP6F and AP6R, 0.5 μM concentrations of the primers AP8NF and AP8NR, 0.25 μM of the primers APXIVA1 and APXIVA3, and 30 to 50 ng of genomic DNA template (isolated by using a Qiagen Mini DNA kit). The following amplification steps were used: 1 cycle of 95°C for 15 min; 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min 30 s; and 1 cycle of 72°C for 7 min.

Initial experiments were performed with genomic DNA from the reference strains, i.e., 4074^T (serovar 1, Argentina), 1536 (serovar 2, Switzerland), S1421 (serovar 3, Switzerland), M62 (serovar 4, United States), K17 (serovar 5a, United States), L20 (serovar 5b, United States), Femø (serovar 6, Denmark), WF83 (serovar 7, Canada), 405 (serovar 8, Ireland), CVJ13261 (serovar 9, The Netherlands), D13039 (serovar 10, Denmark), 56153 (serovar 11, The Netherlands), 8328 (serovar 12, Denmark), N-273 (serovar 13, Hungary), 3906 (serovar 14, Denmark) and HS143 (serovar 15, Australia). The results are shown in Fig. 1. As expected, there was a serovar-specific amplicon of 718 bp in the serovar 6 reference strain (17) and a product of 1,106 bp (the predicted size) in the serovar 8 reference strain. All of the reference strains had an *apxIV* amplicon of 417 bp, as reported previously (53). A serovar-specific amplicon of 921 bp (the predicted size) was only found in the serovar 3 reference strain. The same results (Fig. 1) were found in both the London and Copenhagen laboratories with different types of thermocyclers, different batches of reagents, and template DNAs prepared separately in each individual laboratory. In a small subset of non-*A. pleuropneumoniae* strains there were no amplicons detected (data not shown). Next, the sensitivity of the PCR was determined (Fig. 2). A strong 921-bp serovar 3-specific band was detectable upon ethidium bromide staining with 214 pg of starting genomic DNA template. For serovars 6 and 8, strong bands of 718 and 1,016 bp were visible when 21.4 pg of starting genomic DNA template was used. With all serovars investigated, 21.4 pg was the minimal amount of template DNA required to observe an *apxIV* band.

In view of these promising results, the multiplex PCR test was subsequently applied to our well-characterized large collection of *A. pleuropneumoniae* and non-*A. pleuropneumoniae*

TABLE 1. Field strains used to evaluate the serovar 3, 6, and 8 and *apxIV* multiplex PCR test

Serovar	Country of origin	No. of strains	No. of strains with:			
			apxIV amplicon (417 bp)	Serovar 3 amplicon (921 bp)	Serovar 6 amplicon (718 bp)	Serovar 8 amplicon (1,106 bp)
1	Denmark	1	1	0	0	0
2	Denmark	10	10	0	0	0
	United Kingdom	2	2	0	0	0
3	China	2	2	2	0	0
	Switzerland	9	9	9	0	0
5	Denmark	11	11	0	0	0
6	Denmark	57	57	0	57	0
	United Kingdom	3	3	0	3	0
7	Denmark	8	8	0	0	0
	United Kingdom	5	5	0	0	0
8	China	2	2	0	0	2
	Denmark	53	53	0	0	53
	United Kingdom	10	10	0	0	10
10	Denmark	13	13	0	0	0
12	Denmark	7	7	0	0	0
	United Kingdom	1	1	0	0	0
13	Denmark	2	2	0	0	0
14	Denmark	5	5	0	0	0
Nontypeable	Denmark	49	47[a]	0	0	8[b]

[a] Two K2:O7 strains did not produce an *apxIV* amplicon.
[b] Eight K2:O7 strains had a serovar 8 amplicon. See the text for further details.

TABLE 2. Strains that did not amplify the *apxIV*, serovar 3, 6, or 8 specific gene fragments

Species	Country of origin	Strain(s) designation	No. of strains[a]
Actinobacillus equuli	Denmark	Clinical isolates	3
Actinobacillus genomospecies 1	Denmark	CCUG 22229	1*
	Denmark	CCUG 22231	1*
	Denmark	CCUG 37052	1
Actinobacillus indolicus	Denmark	46KC2^T	1
	Germany	Clinical isolates	10
Actinobacillus lignieresii	United States	ATCC 49236^T	1
	Australia	Clinical isolates	3
	Belgium	Clinical isolates	2
	Denmark	Clinical isolates	27
	Sweden	Clinical isolates	3
	United Kingdom	Clinical isolates	4
	Zimbabwe	Clinical isolate	1
Actinobacillus porcinus	Canada	NM319^T	1
	Germany	Clinical isolates	8
"*Actinobacillus porcitonsillarum*"	Canada	CCUG 46996	1
Actinobacillus minor	Canada	NM305^T	1
	Germany	Clinical isolates	10
Actinobacillus suis	The Netherlands	CAPM 5586^T	1
	Denmark	Clinical isolates	11
Aggregatibacter actinomycetemcomitans	United States	ATCC 43718 (formerly Y4)	1
Bordetella bronchiseptica	United Kingdom	Clinical isolates	2
Escherichia coli	United States	DH5α	1
Haemophilus ducreyi	Canada	35000 (ATCC 33922)	1
Haemophilus paraphrophilus	United Kingdom	NCTC 10557^T	1
Haemophilus parasuis	Unknown	NCTC 4557^T	1
	United Kingdom	Clinical isolate	1
Mannheimia haemolytica	United Kingdom	Clinical isolate	1
Mannheimia varigena	Germany	CCUG 38462^T	1
	Denmark	Clinical isolates	9
Mycoplasma hyopneumoniae	United Kingdom	Clinical isolates	2
Mycoplasma hyosynoviae	United Kingdom	Clinical isolate	1
Mycoplasma hyorhinis	United Kingdom	Clinical isolate	1
Pasteurella multocida	United Kingdom	Clinical isolates	4
Pasteurella pneumotropica	United Kingdom	Clinical isolate	1
Streptococcus suis	United Kingdom	P1/7[b]	1

[a] *, Strains that had an *apxIV* amplicon. See the text for further details.
[b] Langford et al. (22).

strains from China, Denmark, Switzerland, and the United Kingdom (53). All serovar 3 strains were confirmed by toxin typing and/or Southern blotting (2, 10), and serovar 6 and 8 strains were confirmed by serovar-specific PCRs (17, 45). In all experiments, the serovar 3, 6, and 8 reference strains were used as positive controls. With some strains, whole-cell lysates prepared as described previously (17) were used as the source of genomic template DNA. Serovar 3 and 6 *A. pleuropneumoniae* strains had amplicons that were serovar 3 and 6 specific, respectively (Table 1). All serovar 8 *A. pleuropneumoniae* and 8 of 50 nontypeable strains had a serovar 8-specific amplicon. These eight nontypeable strains have been described previously (53), including the presence of a serovar 8 amplicon in the PCR described by Schuchert et al. (45), and belong to a group classified as serovar K2:O7. These rarely isolated genetically heterogeneous (20) biotype 1 strains have capsular polysaccharides and lipopolysaccharides similar to serovars 2 and 7, respectively (36). In contrast to all of the other *A. pleuropneumoniae* strains, two of the K2:O7 strains failed to produce an *apxIV* amplicon (417 bp). Whether the lack of an *apxIV* amplicon in these K2:O7 strains results from sequence divergence in a primer binding site(s) or truncation or lack of the *apxIV* gene was not investigated further. The in vivo specific nature of ApxIV expression (44) precluded investigation as to whether the toxin was expressed in these strains. In some serotype 7 strains, AP76 insertion of the transposable element IS*Apl1* prevents ApxIV-based serological detection (49). Theoretically, insertion of IS*Apl1* or other insertion elements could also lead to PCR failure. No serovar 3, 6, or 8 amplicons were found in any of the non-*A. pleuropneumoniae* strains, which include all of the major bacterial respiratory pathogens and commensals of pigs (Table 2). A large collection of *Actinobacillus lignieresii* strains from diverse origins were screened since the bacterium is the most closely related to *A. pleuropneumoniae* as determined by 16S rRNA sequencing (8) and immunological cross-reactions between *A. pleuropneumoniae* serovars 3, 4, and 7 are known to occur (5, 23). As in our previous study (53), two strains of *Actinobacillus* genomospecies 1 (8) had an *apxIV* amplicon, although they did not PCR amplify serovar 3-, 6-, or 8-specific bands.

In summary, we have designed a single-tube *apxIV*, serovar 3, 6, and 8 multiplex PCR. In total, we screened 16 reference and 254 field strains of *A. pleuropneumoniae* and 120 non-*A. pleuropneumoniae* strains. The test is highly sensitive and specific and is significantly better than our previously described *apxIV* serovar 3-specific PCR since it allows unequivocal determination of the problematic immunologically cross-reactive serovar 3, 6, and 8 strains in a single reaction. The test will be invaluable in epidemiological studies in countries where serovar 3, 6, or 8 strains are found (25 of 27 countries listed in a review of worldwide seroprevalence) (9), especially those where these 3 serovars are among the most prevalent. Such countries include Belgium, Brazil, Hungary, Ireland, and the United Kingdom (9).

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