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1 **Development of a multiplex PCR test for identification of**
2 ***Actinobacillus pleuropneumoniae* serovars 1, 7, and 12**

3
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7

8 **Running title:**

9 Multiplex PCR for *A. pleuropneumoniae* serovars 1, 7, and 12

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17 **ABSTRACT**

18 A PCR assay for simultaneous species identification and separation of *A. pleuropneumoniae*
19 serovars 1, 7 and 12 was developed. Primers specific for genes involved in biosynthesis of
20 the capsular polysaccharides (*cps* genes) of serovars 1, 7, and 12 were combined with a
21 species-specific PCR test based on the *omlA* gene. The PCR test was evaluated with the
22 serovar reference strains of *A. pleuropneumoniae* as well as 183 Danish field isolates. For all
23 typable strains, a complete correspondence was found between results obtained with the
24 multiplex PCR test and results from the traditional serotyping methods. Among 8
25 serologically cross-reacting strains designated K1:O7, seven isolates produced amplicons of
26 similar sizes as serovar 1 and one isolate produced amplicons of similar sizes as serovar 7.
27 The species specificity of the assay was evaluated using a collection of 126 strains
28 representing 25 different species within the family *Pasteurellaceae* including 45 field strains
29 of the phylogenetically affiliated species *Actinobacillus lignieresii*. All these isolates tested
30 negative for the *cps* genes by the multiplex PCR test except for 6 isolates of *A. lignieresii*.
31 Five of these isolates produced an amplicon identical to the *cps* gene of serovar 7, whereas
32 one isolate produced an amplicon identical to the *cps* gene of serovar 1. In addition, four
33 isolates of *Actinobacillus* genomospecies 1 tested positive for the *omlA* gene but negative for
34 the *cps* genes. The test represents a convenient and specific method for serotyping *A.*
35 *pleuropneumoniae* in diagnostic laboratories.

36 **1. Introduction**

37

38 *Actinobacillus pleuropneumoniae* is an encapsulated respiratory pathogen of swine and the
39 causative agent of porcine pleuropneumonia (Gottschalk and Taylor, 2006). At present, 15
40 different serovars and two biotypes have been described (Fodor et al., 1989; Perry et al.,
41 1990; Nielsen et al., 1997; Blackall et al., 2003), the presence and prevalence of which varies
42 among countries. The serovar specificity is predominantly due to structural differences in the
43 capsular polysaccharides (Perry et al., 1990). Studies indicate considerable difference in
44 virulence between serovars (Dom and Haesebrouck, 1992; Jacobsen et al., 1996) .

45

46 A number of different assays have been developed for serotyping of *A. pleuropneumoniae*,
47 but cross-reactions between serovars are often seen in serological assays (Mittal, 1990; Mittal
48 and Bourdon, 1991). Definitive typing of such isolates has been achieved by using more time
49 consuming procedures as immunodiffusion or indirect hemagglutination (Nielsen and
50 O'Connor, 1984). During the last years, the genes involved in biosynthesis of the capsular
51 polysaccharides (*cps* genes) have been described for a number of serovars (Lo et al., 1998;
52 Jessing et al., 2003, 2008; Schuchert et al., 2004; Zhou et al., 2008a, 2008b). PCR assays
53 based on the *cps* genes have proved useful for determining the serovar of *A.*

54 *pleuropneumoniae* isolates. Until now, multiplex PCR tests have been described for serovars
55 2, 5, and 6 (Jessing et al., 2003), and serovars 3, 6, and 8 (Zhou et al., 2008b).

56

57 We have recently described the *cps* genes of serovars 1, 7, and 12 (Jessing et al., 2008). In the
58 present work, a multiplex PCR based on oligonucleotide primers specific for the *cps* region of
59 serovars 1, 7, and 12 combined with primers previously used for species-specific PCR
60 amplification of the *omlA* gene is described (Gram and Ahrens, 1998; Jessing et al., 2003).

61 The specificity of the multiplex PCR test was examined and compared with results obtained
62 from traditional serological typing methods.

63

64 **2. Materials and methods**

65 *2.1. Bacterial strains and growth conditions*

66 All strains used for this study are listed in Table 1 and 2. In addition to reference strains
67 representing serovars 1 to 15, a total of 183 Danish field isolates of *A. pleuropneumoniae*
68 isolated from lungs, representing serovars 1 (n = 28), 2 (n = 5), 5 (n = 5), 6 (n = 5), 7 (n = 42),
69 8 (n=5), 12 (n = 72), K1:O7 (n=8), K2:O7 (n=5), and NT (n=8) were included in the study. In
70 addition, 126 strains representing 25 related species were used to evaluate the species
71 specificity of the multiplex PCR test. All V-factor dependent strains were grown on PPLO
72 agar (Difco, Detroit Mich.), whereas all other strains were grown on Columbia agar
73 supplemented with 5% bovine blood. All strains were incubated at 37°C over night in
74 atmospheric air.

75

76 *2.2 Serotyping of A. pleuropneumoniae*

77 All the Danish field isolates of *A. pleuropneumoniae* were serotyped using a latex
78 agglutination test (Giese et al., 1993). Each isolate was tested with latex particles coated with
79 polyclonal antibodies produced against whole cells of reference strains of *A.*
80 *pleuropneumoniae* representing serovars 1 through 15 (Table 1). Of the 183 Danish field
81 isolates of *A. pleuropneumoniae*, 99 strains could be serotyped by latex agglutination test
82 using polyclonal antibodies to whole cells. The remaining 84 isolates showed cross-reactions
83 between two or more serovars in the latex agglutination test. The serovars of 76 of these
84 isolates were identified by immunodiffusion or indirect haemagglutination (Nielsen and
85 O'Connor, 1984). For 8 strains the serovar could not be determined by any of these methods.

86

87 *2.3 Oligonucleotide primers for PCR*

88 The sequences for the oligonucleotide primers used in this study are listed in Table 3. Four
89 different pairs of primers were used in the multiplex PCR test. Serovar specific primers were
90 designed from the *cps* genes of serovars 1, 7, and 12 (Jessing et al., 2008). These three serovar
91 specific PCR tests were combined with primers used in a previously published species-
92 specific PCR test based on amplification of the *omlA* gene (Gram and Ahrens, 1998). DNA
93 primers designed in this study were selected by using the DNASIS sequence analysis program
94 (Hitachi Software Engineering Co., Ltd.)

95

96 *2.4 Preparation of samples for PCR*

97 Lysates of pure cultures were prepared for all strains used in this study. A 10 µl loopful of an
98 over night culture was taken from the surface of an agar plate and suspended in 200 µl sterile
99 water. The suspension was boiled for 10 min and the debris was pelleted at 13,000g for 2 min.
100 A 180 µl sample of the supernatant was kept for analysis. The lysates were used undiluted as
101 DNA template in the PCR.

102

103 *2.5 PCR amplification*

104 PCR was performed in a total volume of 50 µl containing 10 mM Tris-HCl (pH 8.3); 1.5 mM
105 MgCl₂; 50 mM KCl; 0.005% Tween 20; 0.005% Nonidet P-40 detergent; 100 µM (each)
106 dATP, dCTP, dGTP, and dTTP and 0.5 unit Taq polymerase (Perkin-Elmer). Primers (Table
107 3) were added to a final concentration of 0.1 µM except for Ap12F/Ap12R where 0.3 µM was
108 used. To each reaction, one µl undiluted template DNA was added. Finally, mineral oil was
109 added to prevent evaporation. The PCR reactions were performed in a Biometra Trio
110 thermocycler using 0.5 ml tubes. DNA was amplified for 33 cycles using the following

111 settings: denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extension at 72°C
112 for 1 min. Twelve µl of each reaction was analysed by electrophoresis in a 2% agarose gel.
113 The PCR products were stained with ethidium bromide (10 µg/ml) and visualized under UV
114 light.

115

116 *2.6 Sequencing of PCR products*

117 Sequencing of PCR products was done with an ABI377 automatic sequencer using the Prism
118 BigDye terminator cycle sequencing kit (Applied Biosystems, Foster city, CA). Sequences
119 were compared using DNASIS (Hitachi Software Engineering Co, Ltd.) and homology
120 searches of the GenBank databases were performed using the BLAST server at the National
121 Center for Biotechnology Information.

122

123 **3. Results and discussion**

124 The multiplex PCR was optimized using the serovar reference strains. Primers Ap1F and
125 Ap1R were designed to produce a 754 bp PCR fragment from serovar 1, primers Ap7F and
126 Ap7R a 396 bp PCR fragment from serovar 7, whereas the primer pair Ap12F and Ap12F
127 amplified a 559 bp fragment from serovar 12. These three primer pairs were combined with a
128 species-specific PCR test producing a 950 bp amplicon from all *A. pleuropneumoniae* strains
129 (Gram and Ahrens, 1998).

130

131 The serovar specificity of the multiplex PCR was determined by applying the test to a
132 collection of 199 *A. pleuropneumoniae* isolates, including the serovar reference strains (Table
133 1). For all *A. pleuropneumoniae* strains the species-specific fragment of 950 bp was
134 amplified. For strains belonging to serovars 1, 7, and 12 an additional serovar specific
135 fragment of the expected size was amplified (Figure 1). All Danish field isolates of *A.*

136 *pleuropneumoniae* typed as serovars 1, 7, or 12 with one of the traditional serotyping methods
137 were allocated to the same serovar in the multiplex PCR test. Eight strains that in all
138 serological tests showed cross-reactions to both serovar 1 and 7, were designated K1:O7
139 (Gottschalk et al., 2000). Seven of these strains produced an amplicon of similar size as
140 serovar 7 strains and one isolate produced an amplicon of similar size as serovar 1. A recently
141 published investigation on the genetic diversity of *A. pleuropneumoniae* using AFLP
142 (Kokotovic and Angen, 2007) showed that these 8 strains represent two genetically distinct
143 subpopulations within the species.

144

145 The species specificity of the PCR assay was evaluated with reference strains representing 25
146 different species within the family *Pasteurellaceae* (Table 2). The species-specific primers
147 did not amplify the *A. pleuropneumoniae*-specific amplicon of 950 bp, except for four isolates
148 belonging to *Actinobacillus* genomospecies 1. These strains have been isolated from horses
149 (Christensen et al., 2002) and represent the only described example until now where the
150 *omlA*-based PCR gives an amplicon for strains belonging to other species than *A.*
151 *pleuropneumoniae* and is not likely to cause any problem in a diagnostic laboratory.

152

153 The serovar-specific primers did not amplify any product from any of the 126 heterologous
154 strains tested in this study except for 6 strains belonging to *A. lignieresii*. One of these strains
155 produced an amplicon of similar size as serovar 1 and five isolates produced amplicons of
156 similar sizes as serovar 7. The amplicons were sequenced and were found to be identical to
157 the corresponding *cps* genes of serovars 1 and 7, respectively. *A. lignieresii* is
158 phylogenetically the most affiliated species to *A. pleuropneumoniae* as determined by
159 comparison of the 16S rRNA sequences (Dewhirst et al., 1992). Serological cross-reactions
160 between these two species have earlier been reported for serovar 4 and 7 (Lebrun et al., 1999).

161 The five *A. lignieresii* strains that produced amplicons identical to the *cps* gene sequence of *A.*
162 *pleuropneumoniae* serovar 7 were all serologically typable as serovar 7 using latex
163 agglutination. On the other hand, the strain that produced an amplicon identical to the *cps*
164 gene sequence in serovar 1 was serologically untypable. Serological cross-reaction to *A.*
165 *pleuropneumoniae* serovar 1 has earlier been reported for strains closely affiliated to *A. minor*
166 (Gottschalk et al., 2003). Although several investigations have shown that the serovar of *A.*
167 *pleuropneumoniae* is determined by the *cps* genes carried by the isolates (Lo et al., 1998;
168 Jessing et al., 2003, 2008; Schuchert et al., 2004; Zhou et al., 2008a, 2008b), this shows that
169 these genes can also be found outside the traditionally recognized serovars of *A.*
170 *pleuropneumoniae*. In this investigation we have found *cps* genes similar to serovars 1 and 7
171 both in genetically distinct subpopulations within *A. pleuropneumoniae* as well as in certain
172 strains of *A. lignieresii*. This could suggest that horizontal transfer has been part of the
173 evolution of *A. pleuropneumoniae* capsule forming genes. This is supported by the
174 observation that the *cps* genes have a lower G + C content than the rest of the genome
175 (Jessing et al., 2008).

176

177 In conclusion, the results obtained in this study indicate that the multiplex PCR test is a
178 sensitive, specific, and highly effective diagnostic tool for simultaneous identification and
179 serotyping of *A. pleuropneumoniae* serovars 1, 7, and 12. If this test is used in combination
180 with the earlier described multiplex PCR test (Jessing et al., 2003; Zhou et al., 2008b), simple
181 and serovar specific tests are now available for *A. pleuropneumoniae* serovars 1, 2, 3, 5, 6, 7,
182 8, and 12. These tests represent considerable advantage for diagnostic laboratories by
183 providing quick and reliable serotyping of *A. pleuropneumoniae* isolates and avoiding
184 problems related to conventional serotyping, i.e. variability in typing sera and antigen
185 presentation on cells and the occurrence of serologically cross-reacting strains. Serotyping by

186 PCR also represent a powerful technique for detecting different serovars of *A.*
187 *pleuropneumoniae* from mixed bacterial cultures. It has earlier been applied to detect the
188 presence of different *A. pleuropneumoniae* serovars from swine tonsils (Angen and Jessing,
189 2004) and will probably find application in future epidemiological studies and investigations
190 of carrier animals (Angen et al., 2008). However, the existence of serovar-specific *cps*
191 operons both in genetically distinct subpopulations within the species as well as in other
192 affiliated species can in some cases make the interpretation of the PCR results difficult. In
193 such cases isolation of the bacterium and further genetic characterization will still be
194 necessary.

195

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200 **Legends to Figures**

201 Fig. 1. Agarose gel electrophoresis of DNA fragments generated by multiplex PCR using the
202 reference strains of serovar 1-15 of *A. pleuropneumoniae* (Table 1). For serovars 2 and 12 an
203 additional field strain was included. All strains give rise to a species-specific band of
204 approximately 950 bp in addition to serovar specific bands: 754 bp (serovar 1), 396 bp
205 (serovar 7) and 559 bp (serovar 12). N = non template control, M = DNA molecular weight
206 marker VI (Boehringer Mannheim).

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287 Table 1.
 288 *Actinobacillus pleuropneumoniae* reference strains and field isolates used for evaluation of
 289 the PCR test

<i>Actinobacillus pleuropneumoniae</i>	Strain designation	No. of strains	Amplicon size (bp) ^a
Reference strains			
Serovar 1	Shope 4074 ^T	1	950 + 754
Serovar 2	S 1536	1	950
Serovar 3	S 1421	1	950
Serovar 4	M62	1	950
Serovar 5A	K17	1	950
Serovar 5B	L20	1	950
Serovar 6	Femø	1	950
Serovar 7	WF83	1	950 + 396
Serovar 8	405	1	950
Serovar 9	CVJ 13261	1	950
Serovar 10	D 13039	1	950
Serovar 11	56153	1	950
Serovar 12	8329	1	950 + 559
Serovar 13 (biovar 2)	N-273	1	950
Serovar 14 (biovar 2)	3906	1	950
Serovar 15	HS143	1	950
Danish fields strains			
Serovar 1		28 (16) ^b	950 + 754
Serovar 2		5	950
Serovar 5		5	950
Serovar 6		5	950
Serovar 7		42 (32)	950 + 396
Serovar 8		5	950
Serovar 12		72 (15)	950 + 559
Serovar K2:O7		5	950
Serovar K1:O7		7	950 + 754
Serovar K1:O7		1	950 + 396
Non typable (NT)		8	950

290

291 ^a Amplicon sizes calculated from gene sequences of reference serovar strains.

292 ^b The values in parentheses indicate the number of strains cross-reacting by latex
 293 agglutination.

294

295

296

297

298 Table 2.

299 Genetically affiliated strains used for testing the species specificity of the multiplex PCR test

Species	Strain designation
<i>Actinobacillus lignieresii</i> ^a	ATCC 49236 ^T + 45 field strains
<i>Actinobacillus urea</i>	NCTC 10219 ^T , P1144
<i>Actinobacillus capsulatus</i>	NCTC 11408 ^T , P1364
<i>Actinobacillus hominis</i>	NCTC 11529 ^T , P1336
<i>Actinobacillus equuli</i>	NCTC 8529 ^T , P1284
<i>Actinobacillus suis</i>	CCM 5586 ^T , P1143
<i>Actinobacillus rossii</i>	ATCC 27072 ^T
<i>Actinobacillus genomospecies 1</i> ^b	CCUG 22229, CCUG 22230, CCUG 22231, CCUG 37052, T354/87, C5309b
<i>Actinobacillus minor</i>	NM 305 ^T , T1, T5, T7, 595, JF2280 ^c
<i>Actinobacillus porcicus</i>	NM 319 ^T
<i>Actinobacillus indolicus</i>	46 KC2 ^T
<i>Pasteurella multocida</i>	NCTC 10320, 5, 25, HIM 746-6, F104
<i>Pasteurella canis</i>	CCUG 12400 ^T
<i>Pasteurella aerogenes</i>	ATCC 27883 ^T
<i>Pasteurella stomatis</i>	CCUG 17979 ^T
<i>Pasteurella mairii</i>	CCUG 27189 ^T
<i>Pasteurella langaa</i>	CCUG 15566 ^T
<i>Pasteurella sp. B</i>	CCUG 19794
<i>Mannheimia haemolytica</i>	NCTC 9380 ^T
<i>Mannheimia varigena</i>	CCUG 38462 ^T + 37 field strains
<i>Aggregatibacter actinomycetemcomitans</i>	CCUG 13277 ^T
<i>Haemophilus parasuis</i>	NCTC 4557 ^T
<i>Haemophilus paraphrophilus</i>	3718
<i>Haemophilus segnis</i>	CCUG 10787 ^T
<i>Haemophilus parainfluenzae</i>	CCUG 12836 ^T

300 ^a Five isolates of *A. lignieresii* produced an amplicon identical to *A. pleuropneumoniae*
301 serovar 7 and one isolate produced an amplicon identical to serovar 1.302 ^b Four isolates of *Actinobacillus* genomospecies 1 produced an amplicon of approximately
303 950 bp304 ^c Reference strain of “*Actinobacillus porcitosillarum*”

305

306 Table 3

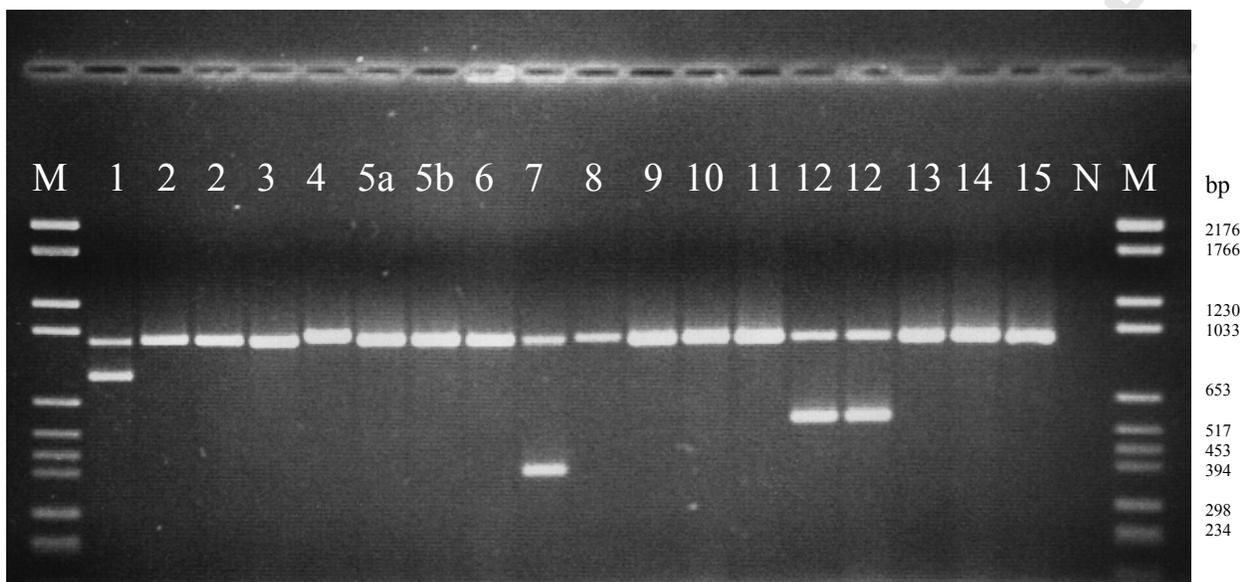
307 Primers used in multiplex PCR test for simultaneous identification and serotyping of *A.*308 *pleuropneumoniae* serovars 1, 7, and 12.

Primer	Function	Sequence 3'-5'	Amplicon size (bp)
HPF	<i>omlA</i> -gene	AAG GTT GAT ATG TCC GCA CC	950
HPR	<i>omlA</i> -gene	CAC CGA TTA CGC CTT GCC A	
Ap1F	<i>cps</i> -region serovar 1	GGG CAA GCC TCT GCT CGT AA	754
Ap1R	<i>cps</i> -region serovar 1	GAA AGA ACC AAG CTC CTG CAA T	
Ap7F	<i>cps</i> -region serovar 7	GGT GAC TGG CGT ACG CCA AA	396
Ap7R	<i>cps</i> -region serovar 7	GGG CTG CAG ACT GAC GTA A	
Ap12F	<i>cps</i> -region serovar 12	GGT TCT CCA GAT GAC TCT GAA A	559
Ap12R	<i>cps</i> -region serovar 12	GCT ATT GGA TGA AGA TGA CTC AT	

309

310

Figure 1



Accepted