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## Accepted Manuscript

Title: Genetic diversity of porcine *Pasteurella multocida* strains from the respiratory tract of healthy and diseased swine

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20 **Abstract**

21 A total of 382 porcine *Pasteurella multocida* strains, isolated from cases of pneumonia  
22 and progressive atrophic rhinitis (PAR) as well as from clinically healthy pigs of more  
23 than 150 German husbandries were characterized by detection of virulence associated  
24 genes (VAGs) and ribotyping to understand the relationships between “commensal” and  
25 “pathogenic” strains, enabling a rational choice of vaccine strains. The diversity of the  
26 strains according to VAGs was low and mainly limited to capsular type genes (*capA*:  
27 53.4%; *capD*: 45.8%; *capF*: 0.3%; *cap*-negative: 0.5%; *hssB*: 95.3%), dermonecrotxin  
28 gene *toxA* (3.4%), as well as adhesion-related genes *pfhaB* (20.9%) and *hgbB* (84.3%).  
29 Ribotyping identified 13 patterns, but the vast majority of strains (95.8%) clustered in  
30 only three of these, namely IA-1 (45.5%), IA-7 (30.1%), and IIA-1 (20.2%). Pattern IA-  
31 1 was associated with *capD*<sup>+</sup> strains (93.6%) and harboured the majority of *toxA*<sup>+</sup> strains  
32 (84.6%). Pattern IA-7 mostly contained *pfhaB*<sup>-</sup>, *toxA*<sup>-</sup> *capA*<sup>+</sup> strains (93.9%), while pat-  
33 tern IIA-1 was predominantly composed of *pfhaB*<sup>+</sup>, *toxA*<sup>-</sup> *capA*<sup>+</sup> strains (87.0%). Clini-  
34 cal strains associated with pneumonia or PAR shared the above mentioned major ribo-  
35 types in comparable proportions with strains derived from healthy pigs, suggesting  
36 *P. multocida* to act more as an opportunistic than as an obligate pathogen in pigs.  
37 The limited number of subpopulations may either reflect a recent evolution of  
38 *P. multocida* in pigs or a selection by means of horizontal transfer of capsular genes,  
39 *toxA* or *pfhaB*. These data enforce further phylogenetic and epidemiological studies,  
40 examining the properties of different subpopulations of porcine *P. multocida* strains as  
41 well as factors of the porcine hosts themselves, which might be involved in disease sus-  
42 ceptibility.

43 **Keywords:** *Pasteurella multocida*; porcine respiratory tract; VAG; PCR; ribotyping

44

## 45 1. Introduction

46 *P. multocida* is part of the normal flora as well as the causative agent of a wide range of  
47 infections of high economic impact. In swine, it causes progressive atrophic rhinitis  
48 (PAR) (Chanter et al., 1989) and is thought to play a crucial role in pneumonia, often  
49 complicating primary infections, e.g. caused by *M. hypopneumoniae* (Ciprian et al.,  
50 1988; Zhao et al., 1992). Although porcine *P. multocida* infections are of great rele-  
51 vance, only little is known about the phylogeny and virulence gene pattern of porcine  
52 strains, particularly about the association between pathogenic isolates, responsible for  
53 pneumonic pasteurellosis and PAR, and strains of the normal flora. Toxin producing  
54 strains associated with PAR are most frequently of capsular type D and less often of  
55 capsular type A (Amigot et al., 1998; Bowersock et al., 1992), while strains causing  
56 pneumonia are usually non-toxigenic and can be of capsular types A and D, the propor-  
57 tion of type A strains usually being higher (Choi et al., 2001; Ewers et al., 2006). Com-  
58 parative genotyping studies of *P. multocida* recovered from PAR and pneumonia, but  
59 also from the normal flora of clinically healthy pigs, have not yet been performed.  
60 Moreover, novel VAGs have been described for *P. multocida*, which, according to a  
61 previously performed study, should be considered in addition to the commonly investi-  
62 gated capsular type genes and the dermonecrotxin gene (Ewers, 2004; Ewers et al.,  
63 2006). The aim of the present study was therefore to comprehensively characterize por-  
64 cine strains of *P. multocida* recovered from cases of pneumonia and PAR as well as  
65 from clinically healthy pigs by comparative analysis through phylotyping and typing of  
66 virulence gene patterns to gain deeper insight into the current *P. multocida* population,  
67 to better understand the relationship between “commensal” and “pathogenic” strains,  
68 and to be able to recommend epidemiologically well-defined vaccines in the future.

## 69 2. Material and Methods

### 70 2.1. Bacterial strains and growth conditions

71 A total of 382 *P. multocida* strains isolated from the respiratory tract of swine were used  
72 in this study, including 205 strains from the upper respiratory tract (URT; nose, conches  
73 and tonsillae), 157 strains from the lower respiratory tract (LRT; tracheobronchial re-  
74 gion and lung) and 20 strains from the respiratory tract without detailed tissue informa-  
75 tion. The majority of strains (n = 361) was obtained from seven different German insti-  
76 tutions (Federal agency of consumer protection (BVL), Veterinary investigation offices,  
77 Institute for Veterinary Pathology Leipzig, German Hybrid Pig Breeding Programme,  
78 IDT Biologika Dessau-Rosslau). The strains were recovered from diseased swine (n =  
79 189), including 155 cases of porcine pneumonia, 25 cases of PAR or suspected PAR  
80 and nine isolates associated with miscellaneous symptoms. Classification was per-  
81 formed using anamnestic information provided by respective institutions. A similar  
82 number of strains (n = 169) was recovered from clinically healthy swine, whereas no  
83 further information regarding the disease status was obtained for 24 additional strains.  
84 Bacterial strains originated from widespread geographical locations within Germany  
85 over a 5-year period (2001 and 2006). Twenty-one *P. multocida* strains were included  
86 from the strain collection of the Institute of Microbiology and Epizootics (IMT), origi-  
87 nating from porcine organ samples or nose swabs investigated in routine diagnostic  
88 tests. Overall, the 382 strains originated from at least 150 different husbandries, 34  
89 strains were of unknown geographical origin. The number of strains per husbandry dif-  
90 fered from one (n = 94) to 14 (n = 1) strains, thus an excessive sampling of multiple  
91 isolates from one farm was avoided. *P. multocida* reference strains NCTC10322 (*capA*,  
92 *kmt1*, *pfhaB*, *ptfA*, *oma87*, *exbB/tonB*, *nanB*, *hgbA*), NCTC10323 (*capB*, *tbpA*),

93 ATCC12948 (*capD*, *toxA*, *nanH*, *hgbB*), P1235 (*capE*) and P4679 (*capF*) were used as  
94 positive controls for the genes indicated. All strains were cultivated on tryptic soy yeast  
95 extract (TSYE) agar, supplemented with 5% sheep blood, and incubated for 18h at 37°C  
96 and 7% CO<sub>2</sub> atmosphere. The identification of strains was based on isolation in pure  
97 culture and a positive PCR result with a primer set for species specific identification of  
98 *P. multocida* (Townsend et al., 1998). Strains were stored at -80°C in 80% (v/v) glycerol  
99 in brain heart infusion broth until further use.

100

## 101 2.2. DNA preparations

102 DNA for PCR analysis was prepared by a boiling procedure. Bacteria from overnight  
103 cultures were suspended in 200µl deionized water and frozen at -20°C for 10min. Af-  
104 terwards the suspension was heated at 98°C in a water bath for another 10min and then  
105 centrifuged for 10min at 13000 x g in an Eppendorff Centrifuge 5415D (Hamburg;  
106 Germany). The supernatant was transferred into a clean 0.5ml Eppendorf tube and  
107 stored at +4°C until use.

108 DNA for ribotyping analysis and probe synthesis was extracted using the Master Pure™  
109 Genomic DNA Purification Kit (Biozym Diagnostik GmbH, Hessisch Oldendorf, Ger-  
110 many) according to the manufacturer's recommendations and stored at -20°C until fur-  
111 ther use. The DNA concentration was determined using spectrophotometer Nanodrop  
112 1000 (Thermo Scientific; Wilmington, USA).

113

## 114 2.3. PCR analyses and gel electrophoresis

115 The capsular types were determined by multiplex capsular PCR typing (Townsend et  
116 al., 2001). Single PCRs for the detection of the *P. multocida* species specific sequence  
117 *kmt1* and various VAGs, including adhesion-related genes *ptfA* and *pfhaB*, iron-related

118 genes *tbpA*, *hgbA*, *hgbB* and *exbB/tonB*, toxin gene *toxA*, outer membrane protein gene  
119 (OMP) *oma87*, and heparosan synthase 2 encoding gene *hssB*, which has been de-  
120 scribed to allow the bacteria changing their capsule components, were performed as  
121 previously described (DeAngelis and White, 2004; Ewers et al., 2006; Townsend et al.,  
122 1998). Briefly, PCR mixtures (25 $\mu$ l) contained 2 $\mu$ l of template DNA, 0.5 $\mu$ l of the four  
123 deoxynucleoside triphosphates (dNTPs) in a primary concentration of 10mM (Rapi-  
124 dozym, Berlin, Germany), 0.1 $\mu$ l of each primer pair in a primary concentration of  
125 100pmol (MWG, Biotech AG, Germany) (Supplemental Table 1); 2.5 $\mu$ l of 10xPCR  
126 buffer, 2.0 $\mu$ l of 50mM MgCl<sub>2</sub>; 0.2 $\mu$ l Taq-Polymerase (1U) (Rapidozym, Berlin, Ger-  
127 many); filled up to the final volume with deionized water. PCR amplification was car-  
128 ried out using a Perkin Elmer thermocycler (Applied Biosystems; GeneAmp PCR Sys-  
129 tem 2400) according to the authors specifications (DeAngelis and White, 2004; Ewers  
130 et al., 2006; Townsend et al., 2001). Amplification products, mixed with 4 $\mu$ l loading  
131 dye, were analysed by gel electrophoresis on a 1% agarose gel, stained with ethidium  
132 bromide and photographed under UV exposure.

133

#### 134 2.4. Restriction endonuclease digestion, Southern Blot and 16SrRNA ribotyping

135 A reaction mixture, containing 3 $\mu$ g DNA, 4 $\mu$ l of 10 x buffer 1 and 5U of restriction  
136 enzyme *HpaII* (New England Biolabs; Frankfurt am Main; Germany) filled to a volume  
137 of 40 $\mu$ l with deionized water, was incubated for 3h at 37°C. After adding 4 $\mu$ l of loading  
138 dye, 25 $\mu$ l of the mixture were transferred onto a 0.7% TAE agarose gel and fragments  
139 were separated overnight (16.5h; 15V) using a standard 1kb size marker (Invitrogen  
140 GmbH; Karlsruhe, Germany) at three positions on each gel to enable a computer-based  
141 normalization of band profiles. The agarose gels were stained with ethidium bromide  
142 and photographed under UV light exposure. Gels were soaked once for 10min in



143 200mMol HCl for acid depurination, followed by 15min washing steps: twice in denatu-  
144 ration solution (1,5M NaCl<sub>2</sub>, 0,5M NaOH) and twice in neutralizing solution (3M  
145 NaCl<sub>2</sub>, 0,5M Tris-HCl; pH7,5). DNA was transferred onto positively charged nylon  
146 membranes (Roche Diagnostics GmbH; Mannheim; Germany) by southern blotting  
147 performed according to a standard protocol (Ausubel, 1999). After DNA transfer, the  
148 membranes were incubated for 30min at 120°C to fix the DNA. 16SrRNA probe was  
149 prepared using strain P4679 and the PCR DIG Probe Synthesis Kit (Roche Diagnostica  
150 GmbH, Germany) according to the manufacture's recommendations. Ribotyping was  
151 performed using the Roche Labelling and Detection Kit (Roche Diagnostics GmbH,  
152 Germany), according to a standard protocol (Ausubel, 1999)

153

#### 154 2.5. Dot blot hybridization analysis

155 To validate the sensitivity of PCR approaches, dot blot hybridization were performed.  
156 The DNA probes for the genes given in parenthesis were prepared with strains  
157 ATCC12945 (*pfhaB*; *hgbB*), ATCC12948 (*toxA*) and NCTC10323 (*tbpA*), using the  
158 PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Germany). Oligonucleotide  
159 primers used for amplification of DNA probes are listed in Supplemental Table 1. Am-  
160 plicons were extracted from the agarose gel using the Agarose Gel DNA extraction kit  
161 (Roche Diagnostics GmbH, Germany). The DNA probe concentration was measured by  
162 fluorescence method (DyNA Quant™ 200, Hoefer, San Francisco, USA) and probes  
163 were stored at -20°C until use. Heat denatured DNA (10 min; 98°C water bath; 10 min  
164 on ice) was transferred onto positively charged nylon membranes, which were incubated  
165 at 120°C for 30min to fix the DNA. Dot blot hybridization was performed using the

166 Roche Labelling and Detection Kit (Roche Diagnostics GmbH, Germany). Hybridiza-  
167 tions were performed according to a standard protocol (Ausubel, 1999).

168

### 169 2.6. Software and statistical analysis

170 The ribotyping pictures were digitalized and analysed using the BioNumerics software  
171 version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). Statistical analyses were  
172 performed using SPSS 15.0 (SPSS GmbH Software; Munich; Germany).

173

## 174 3. Results

### 175 3.1. Distribution of capsular and virulence-associated genes (VAGs)

176 Capsular PCR analysis resulted in the amplification of a single band representing sero-  
177 group specific regions of the biosynthesis loci in all except two isolates. The size of  
178 each PCR fragment corresponded exactly to one of the reference strains A (1,044 bp), D  
179 (657 bp) or F (851 bp) and allowed the capsular type to be determined (Townsend et al.,  
180 2001). As all strains were additionally confirmed as being *P. multocida* in separate PCR  
181 assays using species specific primers (Townsend et al., 1998), the two *cap*-negative  
182 strains were proven to be *P. multocida*. The distribution of capsular types among the  
183 382 porcine *P. multocida* strains is shown in Table 1. More than half of the strains  
184 (53.4%) belonged to capsular type A, 45.8% to capsular type D, one (0.3%) was of cap-  
185 sular type F and two (0.5%) were untypeable. The additional capsular gene *hssB*, coding  
186 for a heparosan synthase 2, was detected in 95.3% of the *P. multocida* strains.

187 Each of the 382 strains harboured *exbB/tonB*, *oma87*, *nanB*, *nanH*, *hgbA* as well as *ptfA*,  
188 while none of them possessed the transferrin binding protein encoding gene *tbpA*. *ToxA*  
189 was detected in only 13 (3.4%) strains. The putative *Pasteurella* filamentous hemagglu-  
190 tinin encoding gene *pfhaB* was present in 20.9% and the haemoglobin binding gene

191 *hgbB* in 84.3% of the strains. Dot Blot hybridization, performed to validate the sensitiv-  
192 ity of PCR approaches, did not identify additional strains positive for *toxA*, *tbpA* and  
193 *pfhaB*, whereas the hybridization against a specific *hgbB* probe revealed additional 14  
194 positive strains that were considered in the results data (Table 1).

195

196 *3.2. Relationship between ribotypes, capsular types, distribution of VAGs, anatomic*  
197 *sites of isolation and clinical history*

198 The distribution of genes with reference to anatomic site of isolation and clinical history  
199 is shown in Table 1. More than half (59.5%) of the 205 URT strains belonged to capsu-  
200 lar type D and less often to capsular type A (40.0%), whereas the majority of 157 LRT  
201 strains (67.5%) were positive for *capA* and less frequently for *capD* (31.2%). The most  
202 prevalent capsular type among *P. multocida* isolated from diseased animals was *capA*  
203 (59.3%), followed by *capD* (39.7%). A nearly similar distribution of these capsular  
204 genes was observed among isolates from clinically healthy pigs. Strains associated with  
205 pneumonia were predominantly of capsular type A (63.9%), whereas those associated  
206 with PAR mostly possessed *capD* (76.0%). The single *capF* positive strain originated  
207 from the lung tissue of a swine suffering from bronchopneumonia.

208 *ToxA* was detected in 2.4% of *P. multocida* from URT and in 3.2% of LRT strains of  
209 both *capA*- (n = 5) and *capD*- (n = 5) positive strains originating from diseased pigs. For  
210 three *toxA* positive strains no information concerning tissue of isolation and disease  
211 status were available. A higher percentage of *toxA* was detected among strains associ-  
212 ated with PAR (16%) compared with strains isolated from cases of pneumonia (3.9%).  
213 All strains harbouring *toxA* were simultaneously positive for *hgbB* and negative for  
214 *pfhaB*. Although there was no highly significant association between the presence of  
215 *pfhaB* and the anatomic site of isolation or clinical history of the strains, the gene was

216 more often detected in LRT (28.7%) compared with URT (15.6%) strains and in strains  
217 isolated from diseased animals (23.4%) compared with those from clinically healthy  
218 pigs (15.3%). Among clinical isolates *pfhaB* was more frequently identified in strains  
219 isolated from pneumonia (24.5%) whereas only 8.0% of the PAR strains harboured this  
220 gene. A significant positive association ( $p<0.001$ ) was calculated for *pfhaB* and *capA*, as  
221 97.5% of *pfhaB* positive strains are of capsule type A, while only one *capD* and one  
222 *capF* strain possessed it. A combination of *pfhaB* and *hgbB* was present in 21 (26.3%)  
223 of the 80 *pfhaB*-positive strains, among them nine from the LRT (eight clinically ill;  
224  $p<0.001$ ). In general, *hgbB* was widespread (84.3%) among the porcine *P. multocida*  
225 strains, exhibiting a significant association ( $p<0.001$ ) with URT (90.2%) but not with  
226 LRT (76.4%) strains. However, there was no significant correlation between the pres-  
227 ence of *hgbB* and the disease status of the host as it was similarly distributed among the  
228 different groups of strains. *HgbB* was regularly detected in capsular type D strains,  
229 while only 70.6% of *capA* positive strains were *hgbB* positive ( $p<0.001$ ).

230 In all but one ribotype pattern (IIA-3) containing multiple isolates, strains from both  
231 clinically healthy and diseased animals were grouped together in comparable propor-  
232 tions (Table 2). In detail, 95.8% of the strains associated with disease were grouped into  
233 ribotype patterns IA-1 (39.7%), IA-7 (35.4%) and IIA-1 (20.6%). Similarly, 97.0% of  
234 strains from clinically healthy pigs belonged to these three patterns. PAR associated  
235 strains were nearly exclusively (88.0%) allocated to ribotype pattern IA-1, whereas  
236 strains isolated from cases of pneumonia were most often found in ribotype pattern IA-7  
237 (41.9%) and in decreasing numbers in pattern IA-1 (32.3%) and IIA-1 (21.3%).

238 Genes differing between the strains, that is, *cap* genes, *toxA*, *pfhaB* and *hgbB*, were  
239 separately grouped in certain patterns (Table 2; Figure 2). There was a clear association

240 ( $p < 0.0001$ ) of certain ribotype patterns with capsule genes *capA* [IA-7 (97.4%); II-1  
241 (94.8%)] and *capD* [IA-1 (93.7%)], while the two *cap*-negative strains were the only  
242 ones forming pattern IA-5. Similarly, most *pfhaB* positive strains (91.3%) were grouped  
243 into cluster II, mainly into pattern IIA-1 ( $p < 0.001$ ) that harboured 83.8% of these. Al-  
244 though not statistically significant, 13 *toxA* positive strains were mainly found in pattern  
245 IA-1 (84.6%). Pattern IA-3 and IA-7 harboured one *toxA* positive strain each.  
246 *HgbB* was found to be widespread among 12 out of 13 ribotype patterns. Notably, the  
247 distribution of *hgbB* among the clustered strains appeared to be contrary to the distribu-  
248 tion of *pfhaB*, in that cluster II, that contained the majority of *pfhaB* positive strains,  
249 included only 34.9% of *hgbB* positive strains, while nearly all strains grouped into the  
250 *pfhaB* negative cluster I contained *hgbB* (98.4%).

251

### 252 3.3. Ribotyping

253 The band patterns resulting from ribotyping analysis of 382 *P. multocida* field and three  
254 reference strains contained six to eight fragments sized between 0.7 and 7.1 kb (Figure  
255 1). According to the similarity of band patterns the strains were grouped into clusters,  
256 sub-clusters and patterns (Figure 2), leading to the identification of clusters I ( $n = 299$ )  
257 and II ( $n = 83$ ), sharing 63.1% similarity. Cluster I was further divided into two sub-  
258 clusters (IA and IB) at a similarity level of 65.7%. The cut-off value for the 13 ribotyp-  
259 ing patterns was set at a similarity level of 95.0%. While sub-cluster IA contained 298  
260 (78.0%) strains distributed among nine ribotype patterns, sub-cluster IB merely con-  
261 sisted of one capsular type D strain originating from the URT. Cluster II comprised 83  
262 (21.7%) strains, which were allocated to ribotype patterns II-1 to II-3. The majority of  
263 the 382 porcine strains (95.8%) was represented by just three ribotype patterns, IA-1

264 (45.5%), IA-7 (30.1%), and IIA-1 (20.2%), whereas the remaining 4.2% were dispersed  
265 among 10 further ribotype patterns (Figure 2).

266 In 12 out of 16 husbandries, from which five or more strains were available, one pattern  
267 was predominant comprising more than 66% of the strains, while in another two farms  
268 one pattern included more than 50% of strains. With a single exception, *P. multocida*  
269 strains from individual federal states were present in more than one ribotype.

270

#### 271 **4. Discussion**

272 Initially, all strains were confirmed as *P. multocida* by two PCRs, one being a  
273 *P. multocida* specific PCR and one specifically detecting capsular biosynthesis genes  
274 (Townsend et al., 2001; Townsend et al., 1998). We detected a higher percentage of  
275 *capA* positive strains associated with pneumonia which is in agreement with previous  
276 studies (Davies et al., 2003; Pijoan et al., 1983; Zhao et al., 1992). Although *capF* posi-  
277 tive and untypeable strains are uncommon in pigs, a few studies verify their infrequent  
278 association with porcine pneumonia (Choi et al., 2001; Davies et al., 2003). The as-  
279 sumption that *capF* positive strains would be adapted to poultry as a host has changed in  
280 the last years, as reports of isolations of serogroup F strains from different animal spe-  
281 cies suffering from respiratory and other symptoms are increasing (Davies et al., 2003;  
282 Ewers et al., 2006; Jaglic et al., 2004). Likewise, the predominance of *capD* positive  
283 (76.0%) strains associated with PAR compared with *capA* positive (24.0%) strains cor-  
284 roborates results of previous studies (Davies et al., 2003; Lariviere et al., 1992). None of  
285 the porcine strains harboured genes coding for capsular type B and E, which are basic-  
286 ally known to be responsible for hemorrhagic septicaemia in cattle and buffalo (Davies et  
287 al., 2003; Rimler, 2000).

288 The overall number of isolates associated with pneumonia (n = 155) compared with  
289 PAR (n = 25) reflects a lower incidence of PAR, which could be due to widespread vac-  
290 cination programs, microbiological surveys of breeding animals and consequent re-  
291 moval of carrier animals. Accordingly, only 13 (3.4%) *P. multocida* strains were posi-  
292 tive for *toxA*, coding for the main etiological factor in the PAR pathogenesis (Lax et al.,  
293 1990). Corresponding to other studies, *toxA* was predominantly (61.5%) detected in, but  
294 not restricted to capsular type D strains. The dermonecrotxin is encoded within a ly-  
295 sogenic bacteriophage (Pullinger et al., 2004), which could account for the low propor-  
296 tion of *toxA* detected in this study due to simple loss of this gene. On the other hand,  
297 phage-mediated transduction also represents a mechanism of horizontal gene transfer  
298 (Davies et al., 2003) and may result in a reintroduction of the *toxA* locus at any time.  
299 The low number of toxin producing strains currently circulating in the field should  
300 therefore not encourage the pig-rearing industry or veterinarians to abandon preventive  
301 strategies.

302 Other genes, coding for products of different functions, including *exxB/tonB* and *hgbA*,  
303 both involved in iron acquisition, neuraminidases (*nanB*, *nanH*), type 4 fimbriae (*ptfA*)  
304 and OMP *oma87*, were regularly detected in all strains tested. In agreement with previ-  
305 ous publications (Ewers et al., 2006; Mizan et al., 2000), a wide distribution of these  
306 genes among *P. multocida* from various host species is substantiated, arguing towards  
307 an increase of fitness and survival of the bacteria within the host environment.

308 Except for capsular types and *toxA*, noticeable differences in the VAG patterns were  
309 determined by the presence or absence of genes coding for the *Pasteurella* filamentous  
310 hemagglutinin (*pfhaB*) and the haemoglobin binding protein B (*hgbB*) (Table 1). *HgbB*  
311 was detected in 90.6% of *P. multocida* from clinically healthy, but in only 80.9% of

312 isolates from diseased swine. Based on sequence analysis and phenotypic assays, *hgbB*  
313 has been characterized as an OMP, being genetically and functionally similar to TonB-  
314 dependent proteins (Cox et al., 2003). Inactivation of HgbB does not affect the ability to  
315 bind haemoglobin, nor does it influence bacterial virulence in a mouse model, suggest-  
316 ing that multiple, alternatively inducible receptors would be beneficial to the bacteria  
317 either by providing an increased level of haemoglobin or by preventing negative effects  
318 of spontaneous mutations in receptor genes (Bosch et al., 2004; Cox et al., 2003). The  
319 absence of *hgbB* may therefore be compensated by systems like *exxB/tonB* and *hgbA*,  
320 both present in all strains tested, a hypothesis which should be clarified by future tran-  
321 scriptional analysis.

322 A combination of *toxA* and *pfhaB* was not found in any strain, while *pfhaB* and *capA*  
323 were significantly associated, and *toxA* was more likely to be associated with *capD*. The  
324 positive association of *pfhaB* and *capA* is not likely due to a physical linkage, as the  
325 genes are separated by about 839 kb on the genome of *P. multocida* strain Pm70 (Acc.-  
326 No.AE004439). Hypothetically, a *capA* positive clone may have acquired *pfhaB* by  
327 horizontal gene transfer and achieved an advantage to survive within the host environ-  
328 ment, resulting in a broad distribution of this clone within the porcine *P. multocida*  
329 population. In contrast, *pfhaB* and *hgbB* were negatively associated ( $p < 0.001$ ) and the  
330 distribution of both genes among strains in ribotype pattern IIA-1 appeared reverse to  
331 each other, as depicted in Figure 2. Knowledge on the role of PphaB in the pathogenesis  
332 of *P. multocida* infections is very scarce. The protein shows a significant similarity to  
333 the *Bordetella pertussis* filamentous hemagglutinin (Fha), which has been shown to be  
334 important for the initial colonization of the nasopharynx and trachea in a mouse infec-  
335 tion model (Kimura et al., 1990). Moreover, Fha is a very effective part of the vaccine



336 against human pertussis (Knight et al., 2006). A *P. multocida fhaB2* knockout mutant  
337 lead to a strong attenuation of the ability to colonize the host after intranasal challenge,  
338 while the resistance towards serum complement was not modified (Tatum et al., 2005).  
339 Other studies demonstrated *pfhaB* mutants to be attenuated in a septicaemia mouse  
340 model, suggesting a crucial role in the initial stage of colonization (Fuller et al., 2000).  
341 Although not significant, we identified a higher proportion of *pfhaB* positive strains in  
342 diseased vs. clinically healthy animals (23.4% vs. 15.3%), which underlines its potential  
343 role in the pathogenesis of porcine pneumonia.

344 The *P. multocida* heparosan synthase 2, encoded by *hssB*, is located outside the known  
345 capsule biosynthesis loci and has previously been identified in all of three *capA*, six of  
346 nine *capD* and six of 11 *capF* positive *P. multocida* strains (DeAngelis and White,  
347 2004). To our knowledge no further study has been performed to examine the distribu-  
348 tion of *hssB* among a greater number of *P. multocida*. A high number of strains (95.3%)  
349 harboured the gene, which is supposed to allow the bacteria to switch the composition  
350 of their capsule polysaccharides, mask surface structures and escape host defence  
351 mechanisms (DeAngelis and White, 2004). Our results support the opinion, that the  
352 gene is commonly distributed within the species, giving the possibility of molecular  
353 camouflage for the pathogen. Because of its overall distribution it is likely to represent  
354 the phylogenetically older genetic structure of capsule synthesis, while the longer  
355 known capsular encoding genes were probably gained later in evolution.

356 Ribotyping appears to be a useful tool for investigating genetic relationships of porcine  
357 *P. multocida* (Blackall et al., 2000; Donnio et al., 1999; Fusing et al., 1999). However,  
358 a comparative analysis of ribotyping and extended VAG typing of *P. multocida* associ-  
359 ated with pneumonia or PAR and of commensal porcine strains has not been undertaken

360 so far. To examine the diversity present in German isolates of porcine *P. multocida*, the  
361 strain collection was deliberately selected to be as diverse as possible. Based on ribotyp-  
362 ing analysis, 382 porcine *P. multocida* strains from different geographical regions in  
363 Germany could be grouped into two clusters (I and II), two sub-clusters (IA and IB) and  
364 13 patterns (IA-1 - IA-9; IB-1, and IIA-1 – IIA-3). The overall diversity of the ribotype  
365 patterns was low, since 95.8% of the isolates were represented by only three ribotypes,  
366 IA-1, IA-7, and IIA-1, which agrees with previous studies unravelling a limited genetic  
367 diversity of porcine *P. multocida* strains (Blackall et al., 2000; Bowles et al., 2000;  
368 Davies et al., 2003; Fussing et al., 1999; Zhao et al., 1992). This accumulation of clini-  
369 cal strains in only three ribotypes suggests a high clonality among virulent porcine  
370 strains leading to disease. Strains originating from clinically healthy animals were  
371 grouped into these three major ribotypes in similar proportions. It is well known that  
372 pneumonic pasteurellosis in swine is often the result of a secondary infection integrated  
373 in a disease complex called “*Mycoplasma*-induced respiratory disease”. In this respect,  
374 sharing of ribotypes by isolates from diseased and healthy animals is not surprising. On  
375 the other hand, opportunistic infections caused by enteric bacteria, such as *Escherichia*  
376 *coli*, are often associated with non pathogenic commensal strains of high genetic diver-  
377 sity (White et al., 1990; Whittam, 1995). Our results substantiate the recent hypothesis  
378 that the natural low diversity of commensal strains occupying the nasopharynx of  
379 healthy pigs accounts for the limited genetic diversity of *P. multocida* strains associated  
380 with porcine pneumonia (Davies et al., 2003). At the same time our comparison of  
381 clinical and commensal strains questions another hypothesis, based on the possibility  
382 that the diversity of commensal strains in the nasopharynx is relatively high, implying  
383 that *P. multocida* is not a secondary pathogen but that virulent clones may have a pri-

384 mary role in the pathogenesis of porcine pneumonia. However, this is still an open ques-  
385 tion, as differences in the animal's environment, such as free roaming, healthy farming  
386 or intensive housing, may have an influence on the distribution and variety of porcine  
387 strains.

388 In our study, strains of the LRT were allocated to seven ribotype patterns revealing a  
389 lower genetic diversity compared with strains from the URT, that were grouped in 11  
390 patterns. This might point towards a higher selective pressure that bacteria have to en-  
391 counter in deeper tissues, probably due to more specific host defence mechanisms. This  
392 would indicate the existence of specialized subsets of *P. multocida*, a hypothesis which  
393 is not fully substantiated by our data. This thesis needs to be verified intensely by more  
394 discriminative and accurate phylogenetic tools, i.e. multilocus sequence typing.

395 Strains of ribotype patterns IA-7 and IIA-1 were strongly associated with capsular type  
396 A, whereas isolates of ribotype pattern IA-1 were associated with capsular type D (Ta-  
397 ble 2, Figure 2). Hence, in contrast to other authors we only rarely found mixed ribotype  
398 patterns regarding the capsular types of the strains (Davies et al., 2003). In recent publi-  
399 cations it has been demonstrated or at least hypothesized that different subpopulations  
400 of *P. multocida* may have been evolved by horizontal transfer of *cap* genes and of *toxA*  
401 or by a conversion of *toxA*-encoding phages. In our study, this could have been hap-  
402 pened to those strains exhibiting a virulence and capsular gene pattern typical of a dis-  
403 tinct ribotype pattern but clustering in another. However, our data more likely indicate  
404 an independent evolution of different lineages of strains resulting in the formation of  
405 various subpopulations.

406 In conclusion, this study provides new knowledge on the diversity and virulence gene  
407 properties of German porcine *P. multocida* strains. The association of a small number of

408 ribotype patterns with porcine pneumonia and PAR and the coexistence of these clinical  
409 strains with those of the normal respiratory flora of clinically healthy swine suggests a  
410 limited genetic diversity, in that, only a few dominant *P. multocida* subpopulations con-  
411 stitute the group of opportunistic pathogens. It is noteworthy to remember that all three  
412 major ribotypes contained strains exhibiting different virulence features, mainly regard-  
413 ing their capsular type and the presence or absence of *toxA* and *pfhaB*, which suggests  
414 some kind of specialization of these subpopulations probably for certain host niches or  
415 environments which could be an interesting issue for further investigations. Future vac-  
416 cine strategies against porcine pasteurellosis and PAR should consider strains of the  
417 three major subpopulations to ensure protection against *P. multocida* infections in swine  
418 as best possible.

419

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424

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**Table 1:** Distribution of virulence-associated genes among 382 *P. multocida* strains related to anatomic site of isolation and anamnestic information

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**Table 2:** Origin, anamnesis, PCR and ribotyping results of 382 porcine *Pasteurella multocida* strains

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**Figure 1:** Presentation of thirteen ribotypes observed among 382 porcine *P. multocida* strains after digestion with *HpaII* and 16S rRNA ribotyping; GibcoBRL 1kb DNA ladder was used as molecular weight marker in the first lane.

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**Figure 2:** Dendrogram based on *Hpa*II ribotypes obtained with 382 *P. multocida* strains

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**Table 1:** Distribution of virulence-associated genes among 382 *P. multocida* strains related to anatomic site of isolation and anamnestic information

	Number (percentage) of strains positive for the respective gene and gene combinations								
	<i>capA</i>	<i>capD</i>	<i>capF</i>	<i>cap-neg.</i>	<i>toxA</i>	<i>pfhaB</i>	<i>hgbB</i>	<i>pfhaB+hgbB</i>	<i>hssB</i>
<b>Total number of strains</b>	204 (53.4)	175 (45.8)	1 (0.3)	2 (0.5)	13 (3.4)	80 (20.9)	322 (84.3)	21 (5.5)	364 (95.3)
<b>Anatomic site</b>									
URT (n=205)	82 (40.0)	122 (59.5)*	0 (0)	1 (0.5)	5 (2.4)	32 (15.6)	185 (90.2)*	12 (5.8)	191 (93.2)
LRT (n=157)	106 (67.5)*	49 (31.2)	1 (0.6)	1 (0.6)	5 (3.2)	45 (28.7)	120 (76.4)	9 (5.7)	153 (97.5)
Unknown (n=20)	16 (80.0)	4 (20.0)	0 (0)	0 (0)	3 (15.0)	3 (15.0)	17 (85.0)	0 (0)	20 (100)
<b>Disease status</b>									
Clinically healthy (n=169)	75 (44.4)	94 (55.0)*	0 (0)	1 (0.6)	0 (0)	26 (15.4)	153 (90.5)	10 (5.9)	158 (93.5)
Clinically ill (n=189)	112 (59.3)	75 (39.7)	1 (0.5)	1 (0.5)	10 (5.3)	44 (23.3)	153 (81.0)	8 (4.2)	186 (98.4)
Pneumonia (n = 155)	99 (63.9)	54 (34.8)	1 (0.6)	1 (0.6)	6 (3.9)	38 (24.5)	124 (80.0)	7 (4.5)	155 (100.0)
PAR (n = 25)	6 (24.0)	19 (76.0)	0 (0)	0 (0)	4 (16.0)	2 (8.0)	23 (92.0)	0 (0)	22 (88.0)
Miscellaneous (n = 9)	7 (77.8)	2 (22.2)	0 (0)	0 (0)	0 (0)	4 (44.4)	6 (66.7)	1 (11.1)	9 (100.0)
Unknown (n=24)	17 (70.8)	7 (29.2)	0 (0)	0 (0)	3 (8.0)	10 (42.7)	16 (66.7)	3 (12.5)	20 (83.3)

URT = Upper respiratory tract; LRT = Lower respiratory tract; PAR = Progressive atrophic rhinitis

significant association ( $p < 0.001$ ) with various criteria are indicated by an asterix.

**Table 2:** Origin, anamnesis, PCR and ribotyping results of 382 porcine *Pasteurella multocida* strains

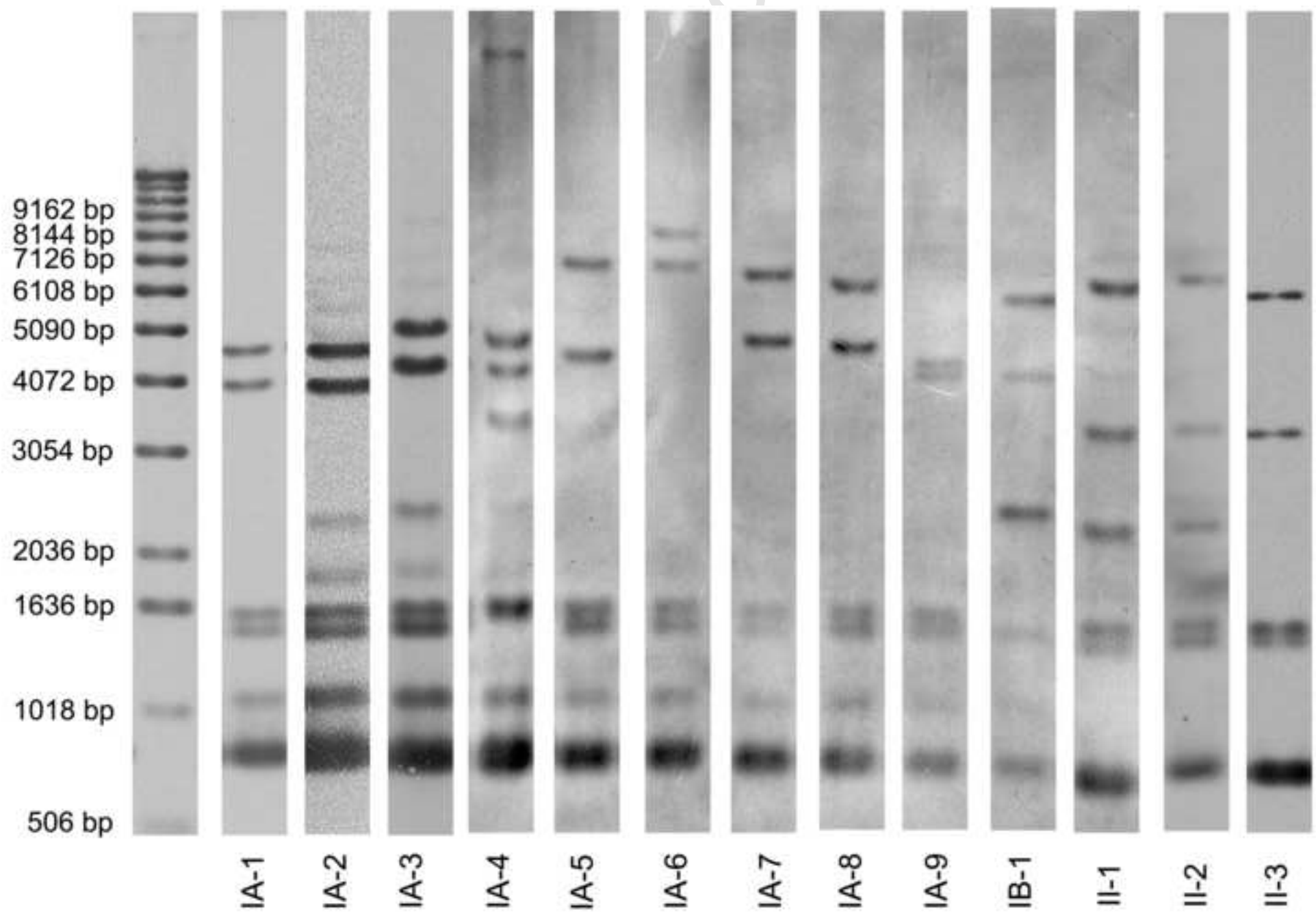
No. of strains	Ribotype patterns			No. of strains per pattern	No. of strains positive for the respective genes									Anamnesis			Anatomic origin of samples			
	Cluster	Sub-cluster	Pattern		<i>capA</i>	<i>capD</i>	<i>capF</i>	<i>cap-neg</i>	<i>toxA</i>	<i>pfhaB</i>	<i>hgbB</i>	<i>hssB</i>	healthy	ill	pneumonia	PAR	unknown	URT*	LRT*	unknown
298	I	A	1	174	11	163	0	0	11	3	171	159	93	75	50	22	6	123	45	6
	I	A	2	1	0	1	0	0	0	0	1	1	1	0	0	0	0	1	0	0
	I	A	3	2	1	1	0	0	1	0	2	2	1	1	0	1	0	2	0	0
	I	A	4	1	0	1	0	0	0	0	1	1	0	1	1	0	0	1	0	0
	I	A	5	2	0	0	0	2	0	0	2	2	1	1	1	0	0	1	1	0
	I	A	6	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0
	I	A	7	115	112	3	0	0	1	4	112	114	43	67	65	0	5	41	63	11
	I	A	8	1	1	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0
	I	A	9	1	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0
1	I	B	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0
83	II	A	1	77	73	4	0	0	0	67	24	75	28	39	33	2	10	32	42	3
	II	A	2	1	1	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0
	II	A	3	5	3	1	1	0	0	5	5	5	0	3	3	0	2	1	4	0
Σ: 382				204	175	1	2	13	80	322	364	169	189	155	25	24	205	157	20	

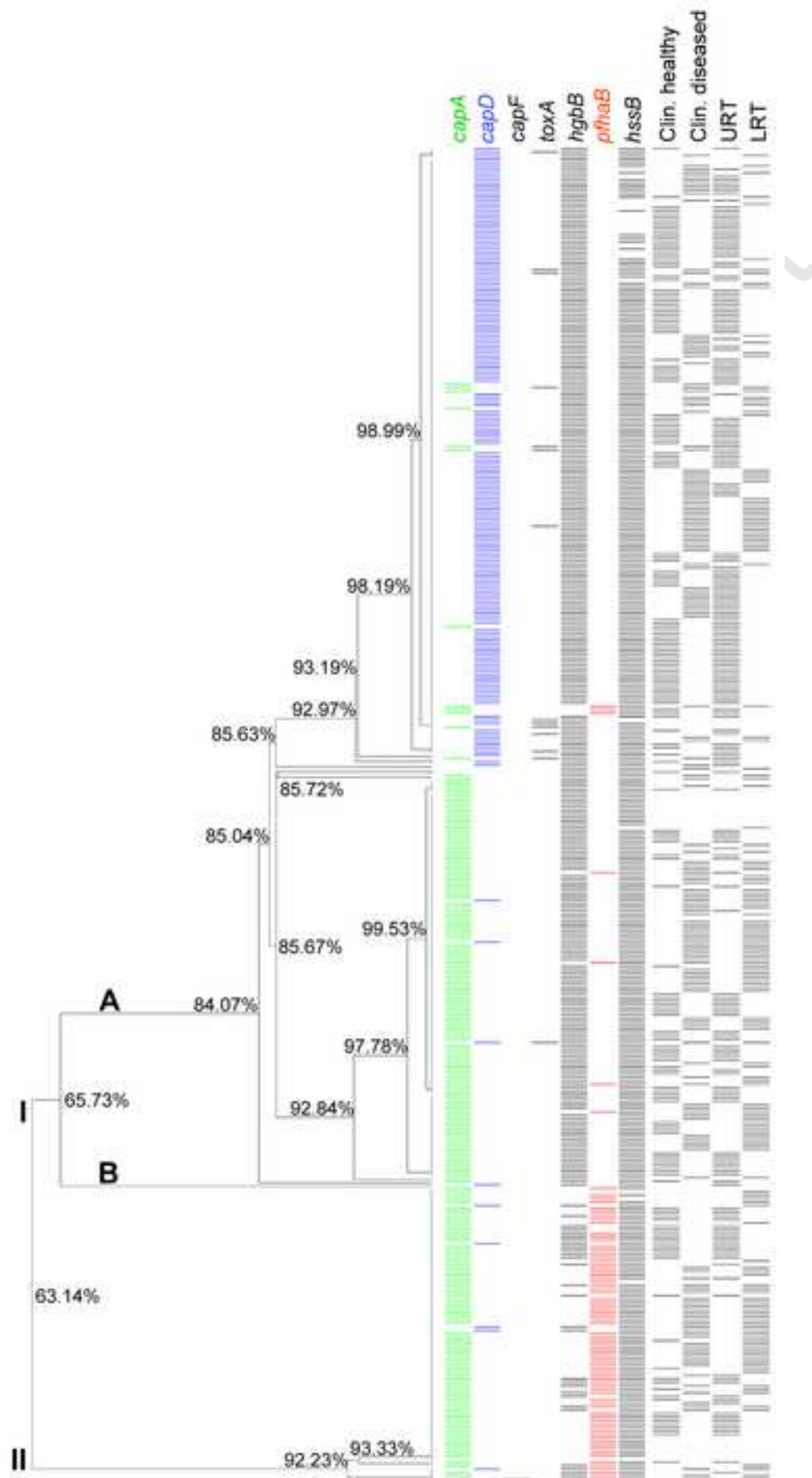
\*URT = Upper respiratory tract, including strains isolated from nasal swabs, conchae and tonsillae;

\*LRT = Lower respiratory tract, including strains isolated from lung tissue and tracheobronchial regions

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Figure1







**Supplemental Table 1:** Sequence, specificity, and PCR conditions applied for the detection of virulence-associated genes in *P. multocida* strains

Primer	Primer sequence (5' - 3')	Detected gene	Product size (bp)	Reference
KMT1 s/ <u>as</u>	ATCCGCTATTTACCCAGTGG/ <u>GCTGTAAACGAACTCGCCAC</u>	<i>kmt1</i>	456	Townsend et al, (2001)
CapA s/ <u>as</u>	TGCCAAAATCGCAGTCAG/ <u>TTGCCATCATTGTCAGTG</u>	<i>hyaD/C</i>	1044	Townsend et al, (2001)
CapB s/ <u>as</u>	CATTTATCCAAGCTCCACC/ <u>GCCCGAGAGTTTCAATCC</u>	<i>bcbB</i>	759	Townsend et al, (2001)
CapD s/ <u>as</u>	TTACAAAAGAAAGACTAGGAGCCC/ <u>CATCTACCCACTCAACCATATCAG</u>	<i>dcbF</i>	657	Townsend et al, (2001)
CapE s/ <u>as</u>	TCCGCAGAAAATTATTGACTC/ <u>GCTTGCTGCTTGATTTTGTC</u>	<i>ecbJ</i>	513	Townsend et al, (2001)
CapF s/ <u>as</u>	AATCGGAGAACGCAGAAATCAG/ <u>TTCCGCCGTCAATTA</u> CTCTG	<i>fc bD</i>	852	Townsend et al, (2001)
Fim4 s/ <u>as</u>	TGTGGAATTCAGCATTTTAGTGTGTC/ <u>TCATGAATTCTTATGCGCAAATCCTGCTGG</u>	<i>ptfA</i>	500	Doughty et al., (2000)
ToxA s*/ <u>as</u> *	CTTAGATGAGCGACAAGGTT/ <u>GGAATGCCACACCTCTATA</u>	<i>toxA</i>	848	Ewers et al. (2006)
ToxA s/ <u>as</u>	ATGAAAACAAAACATTTTTTTAACT/ <u>TTATAGTGCTCTTGTTAAGCGAGGC</u>	<i>toxA</i>	3858	Ewers et al. (2006)
Oma87 s/ <u>as</u>	ATGAAAAAACTTTTAATTGCGAGC/ <u>TGACTTGCGCAGTTGCATAAC</u>	<i>oma87</i>	949	Ewers et al. (2006)
NanB s/ <u>as</u>	GTCCTATAAAGTGACGCCGA/ <u>ACAGCAAAGGAAGACTGTCC</u>	<i>nanB</i>	585	Ewers et al. (2006)
NanH s/ <u>as</u>	GAATATTTGGGCGGCAACA/ <u>TTCTCGCCCTGTCATCACT</u>	<i>nanH</i>	361	Ewers et al. (2006)
ExbB s/tonB <u>as</u>	GGTGGTGATATTGATGCGGC/ <u>GCATCATGCGTGCACGGTT</u>	<i>exbBD/tonB</i>	1144	Ewers et al. (2006)
PfhaB s*/ <u>as</u> *	AGCTGATCAAGTGGTGAAC/ <u>TGGTACATTGGTGAATGCTG</u>	<i>pfhA</i>	275	Ewers et al. (2006)

Primer	Primer sequence (5' - 3')	Detected gene	Product size (bp)	Reference
HgbA s/ <u>as</u>	TGGCGGATAGTCATCAAG/ <u>CCAAAGAACCACTACCCA</u>	<i>hgbA</i>	420	Ewers et al. (2006)
HgbB s*/ <b>as</b> *	ACCGCGTTGGAATTATGATTG/ <u>CATTGAGTACGGCTTGACAT</u>	<i>hgbB</i>	789	Ewers et al. (2006)
TbpA s*/ <u>as</u> *	TTGGTTGGAAACGGTAAAGC/ <u>TAACGTGTACGGAAAAGCCC</u>	<i>tbpA</i>	729	Ewers et al. (2006)
TbpA s/ <b>as</b>	TGCGACAACGGAAATTTCCCTC/GGACAGTGCATATAACTTGTTTACTA	<i>tpbA</i>	2073	This study
16SrRNA s/ <b>as</b>	ATGCAAGTCGAACGGTAGCAGG/ <u>GTGACGGGCGGTGTGTACAA</u>	<i>16SrRNA</i>	1355	This study
Hssb s/ <u>as</u>	ACAGGACAGTAAACATCCGC/ <u>GTTGCTCATCATTGTCTCGAT</u>	<i>hssB</i>	1685	This study

s = sense, as = antisense; \*primer used for PCR; **bold**: primer used for amplification of DNA-probes