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

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Abstract

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

Keywords: Pasteurella multocida; porcine respiratory tract; VAG; PCR; ribotyping
1. Introduction

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

2.1. Bacterial strains and growth conditions

A total of 382 *P. multocida* strains isolated from the respiratory tract of swine were used in this study, including 205 strains from the upper respiratory tract (URT; nose, conches and tonsillae), 157 strains from the lower respiratory tract (LRT; tracheobronchial region and lung) and 20 strains from the respiratory tract without detailed tissue information. The majority of strains (n = 361) was obtained from seven different German institutions (Federal agency of consumer protection (BVL), Veterinary investigation offices, Institute for Veterinary Pathology Leipzig, German Hybrid Pig Breeding Programme, IDT Biologika Dessau-Rossau). The strains were recovered from diseased swine (n = 189), including 155 cases of porcine pneumonia, 25 cases of PAR or suspected PAR and nine isolates associated with miscellaneous symptoms. Classification was performed using anamnestic information provided by respective institutions. A similar number of strains (n = 169) was recovered from clinically healthy swine, whereas no further information regarding the disease status was obtained for 24 additional strains. Bacterial strains originated from widespread geographical locations within Germany over a 5-year period (2001 and 2006). Twenty-one *P. multocida* strains were included from the strain collection of the Institute of Microbiology and Epizootics (IMT), originating from porcine organ samples or nose swabs investigated in routine diagnostic tests. Overall, the 382 strains originated from at least 150 different husbandries, 34 strains were of unknown geographical origin. The number of strains per husbandry differed from one (n = 94) to 14 (n = 1) strains, thus an excessive sampling of multiple isolates from one farm was avoided. *P. multocida* reference strains NCTC10322 (*capA, kmt1, pfhaB, ptfA, oma87, exbB/tonB, nanB, hgbA*), NCTC10323 (*capB, tbpA*),
ATCC12948 (capD, toxA, nanH, hgbB), P1235 (capE) and P4679 (capF) were used as positive controls for the genes indicated. All strains were cultivated on tryptic soy yeast extract (TSYE) agar, supplemented with 5% sheep blood, and incubated for 18h at 37°C and 7% CO₂ atmosphere. The identification of strains was based on isolation in pure culture and a positive PCR result with a primer set for species specific identification of *P. multocida* (Townsend et al., 1998). Strains were stored at -80°C in 80% (v/v) glycerol in brain heart infusion broth until further use.

2.2. DNA preparations

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

DNA for ribotyping analysis and probe synthesis was extracted using the Master Pure™ Genomic DNA Purification Kit (Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany) according to the manufacturer’s recommendations and stored at -20°C until further use. The DNA concentration was determined using spectrophotometer Nanodrop 1000 (Thermo Scientific; Wilmington, USA).

2.3. PCR analyses and gel electrophoresis

The capsular types were determined by multiplex capsular PCR typing (Townsend et al., 2001). Single PCRs for the detection of the *P. multocida* species specific sequence kmt1 and various VAGs, including adhesion-related genes ptfA and pfihaB, iron-related
genes tbpA, hgbA, hgbB and exbB/tonB, toxin gene toxA, outer membrane protein gene
(OMP) oma87, and heparosan synthase 2 encoding gene hssB, which has been de-
scribed to allow the bacteria changing their capsule components, were performed as
previously described (DeAngelis and White, 2004; Ewers et al., 2006; Townsend et al.,
1998). Briefly, PCR mixtures (25µl) contained 2µl of template DNA, 0.5µl of the four
deoxyribonucleotide triphosphates (dNTPs) in a primary concentration of 10mM (Rapi-
dozym, Berlin, Germany), 0.1µl of each primer pair in a primary concentration of
100pmol (MWG, Biotech AG, Germany) (Supplemental Table 1); 2.5µl of 10xPCR
buffer, 2.0µl of 50mM MgCl$_2$; 0.2µl Taq-Polymerase (1U) (Rapidozym, Berlin, Ger-
many); filled up to the final volume with deionized water. PCR amplification was car-
ried out using a Perkin Elmer thermocycler (Applied Biosystems; GeneAmp PCR Sys-
ystem 2400) according to the authors specifications (DeAngelis and White, 2004; Ewers
et al., 2006; Townsend et al., 2001). Amplification products, mixed with 4µl loading
dye, were analysed by gel electrophoresis on a 1% agarose gel, stained with ethidium
bromide and photographed under UV exposure.

2.4. Restriction endonuclease digestion, Southern Blot and 16SrRNA ribotyping
A reaction mixture, containing 3µg DNA, 4µl of 10 x buffer 1 and 5U of restriction
enzyme HpaII (New England Biolabs; Frankfurt am Main; Germany) filled to a volume
of 40µl with deionized water, was incubated for 3h at 37°C. After adding 4µl of loading
dye, 25µl of the mixture were transferred onto a 0.7% TAE agarose gel and fragments
were separated overnight (16.5h; 15V) using a standard 1kb size marker (Invitrogen
GmbH; Karlsruhe, Germany) at three positions on each gel to enable a computer-based
normalization of band profiles. The agarose gels were stained with ethidium bromide
and photographed under UV light exposure. Gels were soaked once for 10min in
200mMol HCl for acid depurination, followed by 15min washing steps: twice in denaturation solution (1,5M NaCl₂, 0,5M NaOH) and twice in neutralizing solution (3M NaCl₂, 0,5M Tris-HCl; pH7,5). DNA was transferred onto positively charged nylon membranes (Roche Diagnostics GmbH; Mannheim; Germany) by southern blotting performed according to a standard protocol (Ausubel, 1999). After DNA transfer, the membranes were incubated for 30min at 120°C to fix the DNA. 16SrRNA probe was prepared using strain P4679 and the PCR DIG Probe Synthesis Kit (Roche Diagnostica GmbH, Germany) according to the manufacture’s recommendations. Ribotyping was performed using the Roche Labelling and Detection Kit (Roche Diagnostics GmbH, Germany), according to a standard protocol (Ausubel, 1999)

2.5. Dot blot hybridization analysis
To validate the sensitivity of PCR approaches, dot blot hybridization were performed. The DNA probes for the genes given in parenthesis were prepared with strains ATCC12945 (pfhaB; hgbB), ATCC12948 (toxA) and NCTC10323 (tbpA), using the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Germany). Oligonucleotide primers used for amplification of DNA probes are listed in Supplemental Table 1. Amplicons were extracted from the agarose gel using the Agarose Gel DNA extraction kit (Roche Diagnostics GmbH, Germany). The DNA probe concentration was measured by fluorescence method (DyNA Quant™ 200, Hoefer, San Francisco, USA) and probes were stored at -20°C until use. Heat denatured DNA (10 min; 98°C water bath; 10 min on ice) was transferred onto positively charged nylon membranes, which were incubated at 120°C for 30min to fix the DNA. Dot blot hybridization was performed using the
Roche Labelling and Detection Kit (Roche Diagnostics GmbH, Germany). Hybridizations were performed according to a standard protocol (Ausubel, 1999).

2.6. Software and statistical analysis

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

3. Results

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

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

Each of the 382 strains harbouring *exbB/tonB, oma87, nanB, nanH, hgbA* as well as *ptfA*, while none of them possessed the transferrin binding protein encoding gene *tbpA. ToxA* was detected in only 13 (3.4%) strains. The putative *Pasteurella* filamentous hemagglutinin encoding gene *pfhaB* was present in 20.9% and the haemoglobin binding gene...
hgbB in 84.3% of the strains. Dot Blot hybridization, performed to validate the sensitivity of PCR approaches, did not identify additional strains positive for toxA, tbpA and pfhaB, whereas the hybridization against a specific hgbB probe revealed additional 14 positive strains that were considered in the results data (Table 1).

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

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

ToxA was detected in 2.4% of P. multocida from URT and in 3.2% of LRT strains of both capA- (n = 5) and capD- (n = 5) positive strains originating from diseased pigs. For three toxA positive strains no information concerning tissue of isolation and disease status were available. A higher percentage of toxA was detected among strains associated with PAR (16%) compared with strains isolated from cases of pneumonia (3.9%). All strains harbouring toxA were simultaneously positive for hgbB and negative for pfhaB. Although there was no highly significant association between the presence of pfhaB and the anatomic site of isolation or clinical history of the strains, the gene was
more often detected in LRT (28.7%) compared with URT (15.6%) strains and in strains
isolated from diseased animals (23.4%) compared with those from clinically healthy
pigs (15.3%). Among clinical isolates pfhaB was more frequently identified in strains
isolated from pneumonia (24.5%) whereas only 8.0% of the PAR strains harboured this
gene. A significant positive association ($p<0.001$) was calculated for pfhaB and capA, as
97.5% of pfhaB positive strains are of capsule type A, while only one capD and one
capF strain possessed it. A combination of pfhaB and hgbB was present in 21 (26.3%)
of the 80 pfhaB-positive strains, among them nine from the LRT (eight clinically ill;
$p<0.001$). In general, hgbB was widespread (84.3%) among the porcine $P.\ multocida$
strains, exhibiting a significant association ($p<0.001$) with URT (90.2%) but not with
LRT (76.4%) strains. However, there was no significant correlation between the pres-
ence of hgbB and the disease status of the host as it was similarly distributed among the
different groups of strains. HgbB was regularly detected in capsular type D strains,
while only 70.6% of capA positive strains were hgbB positive ($p<0.001$).

In all but one ribotype pattern (IIA-3) containing multiple isolates, strains from both
clinically healthy and diseased animals were grouped together in comparable propor-
tions (Table 2). In detail, 95.8% of the strains associated with disease were grouped into
ribotype patterns IA-1 (39.7%), IA-7 (35.4%) and IIA-1 (20.6%). Similarly, 97.0% of
strains from clinically healthy pigs belonged to these three patterns. PAR associated
strains were nearly exclusively (88.0%) allocated to ribotype pattern IA-1, whereas
strains isolated from cases of pneumonia were most often found in ribotype pattern IA-7
(41.9%) and in decreasing numbers in pattern IA-1 (32.3%) and IIA-1 (21.3%).
Genes differing between the strains, that is, cap genes, toxA, pfhaB and hgbB, were
separately grouped in certain patterns (Table 2; Figure 2). There was a clear association
of certain ribotype patterns with capsule genes \textit{capA} [IA-7 (97.4%); II-1 (94.8%)] and \textit{capD} [IA-1 (93.7%)], while the two \textit{cap}-negative strains were the only ones forming pattern IA-5. Similarly, most \textit{pfhaB} positive strains (91.3%) were grouped into cluster II, mainly into pattern IIA-1 \((p<0.001)\) that harboured 83.8% of these. Although not statistically significant, 13 \textit{toxA} positive strains were mainly found in pattern IA-1 (84.6%). Pattern IA-3 and IA-7 harboured one \textit{toxA} positive strain each.

\(HgbB\) was found to be widespread among 12 out of 13 ribotype patterns. Notably, the distribution of \textit{hgbB} among the clustered strains appeared to be contrary to the distribution of \textit{pfhaB}, in that cluster II, that contained the majority of \textit{pfhaB} positive strains, included only 34.9\% of \textit{hgbB} positive strains, while nearly all strains grouped into the \textit{pfhaB} negative cluster I contained \textit{hgbB} (98.4\%).

\subsection*{3.3. Ribotyping}

The band patterns resulting from ribotyping analysis of 382 \textit{P. multocida} field and three reference strains contained six to eight fragments sized between 0.7 and 7.1 kb (Figure 1). According to the similarity of band patterns the strains were grouped into clusters, sub-clusters and patterns (Figure 2), leading to the identification of clusters I \((n = 299)\) and II \((n = 83)\), sharing 63.1\% similarity. Cluster I was further divided into two sub-clusters (IA and IB) at a similarity level of 65.7\%. The cut-off value for the 13 ribotyping patterns was set at a similarity level of 95.0\%. While sub-cluster IA contained 298 (78.0\%) strains distributed among nine ribotype patterns, sub-cluster IB merely consisted of one capsular type D strain originating from the URT. Cluster II comprised 83 (21.7\%) strains, which were allocated to ribotype patterns II-1 to II-3. The majority of the 382 porcine strains (95.8\%) was represented by just three ribotype patterns, IA-1
(45.5%), IA-7 (30.1%), and IIA-1 (20.2%), whereas the remaining 4.2% were dispersed among 10 further ribotype patterns (Figure 2).

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

### 4. Discussion

Initially, all strains were confirmed as *P. multocida* by two PCRs, one being a *P. multocida* specific PCR and one specifically detecting capsular biosynthesis genes (Townsend et al., 2001; Townsend et al., 1998). We detected a higher percentage of *capA* positive strains associated with pneumonia which is in agreement with previous studies (Davies et al., 2003; Pijoan et al., 1983; Zhao et al., 1992). Although *capF* positive and untypeable strains are uncommon in pigs, a few studies verify their infrequent association with porcine pneumonia (Choi et al., 2001; Davies et al., 2003). The assumption that *capF* positive strains would be adapted to poultry as a host has changed in the last years, as reports of isolations of serogroup F strains from different animal species suffering from respiratory and other symptoms are increasing (Davies et al., 2003; Ewers et al., 2006; Jaglic et al., 2004). Likewise, the predominance of *capD* positive (76.0%) strains associated with PAR compared with *capA* positive (24.0%) strains corroborates results of previous studies (Davies et al., 2003; Lariviere et al., 1992). None of the porcine strains harboured genes coding for capsular type B and E, which are basically known to be responsible for hemorrhagic septicaemia in cattle and buffalo (Davies et al., 2003; Rimler, 2000).
The overall number of isolates associated with pneumonia (n = 155) compared with PAR (n = 25) reflects a lower incidence of PAR, which could be due to widespread vaccination programs, microbiological surveys of breeding animals and consequent removal of carrier animals. Accordingly, only 13 (3.4%) *P. multocida* strains were positive for *toxA*, coding for the main etiological factor in the PAR pathogenesis (Lax et al., 1990). Corresponding to other studies, *toxA* was predominantly (61.5%) detected in, but not restricted to capsular type D strains. The dermonecrotxin is encoded within a lysogenic bacteriophage (Pullinger et al., 2004), which could account for the low proportion of *toxA* detected in this study due to simple loss of this gene. On the other hand, phage-mediated transduction also represents a mechanism of horizontal gene transfer (Davies et al., 2003) and may result in a reintroduction of the *toxA* locus at any time. The low number of toxin producing strains currently circulating in the field should therefore not encourage the pig-rearing industry or veterinarians to abandon preventive strategies.

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

Except for capsular types and *toxA*, noticeable differences in the VAG patterns were determined by the presence or absence of genes coding for the *Pasteurella* filamentous hemagglutinin (*pfhaB*) and the haemoglobin binding protein B (*hgbB*) (Table 1). *HgbB* was detected in 90.6% of *P. multocida* from clinically healthy, but in only 80.9% of
isolates from diseased swine. Based on sequence analysis and phenotypic assays, \textit{hgbB} has been characterized as an OMP, being genetically and functionally similar to TonB-dependent proteins (Cox et al., 2003). Inactivation of HgbB does not affect the ability to bind haemoglobin, nor does it influence bacterial virulence in a mouse model, suggesting that multiple, alternatively inducible receptors would be beneficial to the bacteria either by providing an increased level of haemoglobin or by preventing negative effects of spontaneous mutations in receptor genes (Bosch et al., 2004; Cox et al., 2003). The absence of \textit{hgbB} may therefore be compensated by systems like \textit{exbB/tonB} and \textit{hgbA}, both present in all strains tested, a hypothesis which should be clarified by future transcriptional analysis.

A combination of \textit{toxA} and \textit{pfhaB} was not found in any strain, while \textit{pfhaB} and \textit{capA} were significantly associated, and \textit{toxA} was more likely to be associated with \textit{capD}. The positive association of \textit{pfhaB} and \textit{capA} is not likely due to a physical linkage, as the genes are separated by about 839 kb on the genome of \textit{P. multocida} strain Pm70 (Acc.-No.AE004439). Hypothetically, a \textit{capA} positive clone may have acquired \textit{pfhaB} by horizontal gene transfer and achieved an advantage to survive within the host environment, resulting in a broad distribution of this clone within the porcine \textit{P. multocida} population. In contrast, \textit{pfhaB} and \textit{hgbB} were negatively associated (\textit{p}<0.001) and the distribution of both genes among strains in ribotype pattern IIA-1 appeared reverse to each other, as depicted in Figure 2. Knowledge on the role of PfhaB in the pathogenesis of \textit{P. multocida} infections is very scarce. The protein shows a significant similarity to the \textit{Bordetella pertussis} filamentous hemagglutinin (Fha), which has been shown to be important for the initial colonization of the nasopharynx and trachea in a mouse infection model (Kimura et al., 1990). Moreover, Fha is a very effective part of the vaccine
against human pertussis (Knight et al., 2006). A *P. multocida fhaB2* knockout mutant lead to a strong attenuation of the ability to colonize the host after intranasal challenge, while the resistance towards serum complement was not modified (Tatum et al., 2005). Other studies demonstrated *pfhaB* mutants to be attenuated in a septicaemia mouse model, suggesting a crucial role in the initial stage of colonization (Fuller et al., 2000). Although not significant, we identified a higher proportion of *pfhaB* positive strains in diseased vs. clinically healthy animals (23.4% vs. 15.3%), which underlines its potential role in the pathogenesis of porcine pneumonia.

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

Ribotyping appears to be a useful tool for investigating genetic relationships of porcine *P. multocida* (Blackall et al., 2000; Donnio et al., 1999; Fussing et al., 1999). However, a comparative analysis of ribotyping and extended VAG typing of *P. multocida* associated with pneumonia or PAR and of commensal porcine strains has not been undertaken.
so far. To examine the diversity present in German isolates of porcine *P. multocida*, the strain collection was deliberately selected to be as diverse as possible. Based on ribotyping analysis, 382 porcine *P. multocida* strains from different geographical regions in Germany could be grouped into two clusters (I and II), two sub-clusters (IA and IB) and 13 patterns (IA-1 - IA-9; IB-1, and IIA-1 – IIA-3). The overall diversity of the ribotype patterns was low, since 95.8% of the isolates were represented by only three ribotypes, IA-1, IA-7, and IIA-1, which agrees with previous studies unravelling a limited genetic diversity of porcine *P. multocida* strains (Blackall et al., 2000; Bowles et al., 2000; Davies et al., 2003; Fussing et al., 1999; Zhao et al., 1992). This accumulation of clinical strains in only three ribotypes suggests a high clonality among virulent porcine strains leading to disease. Strains originating from clinically healthy animals were grouped into these three major ribotypes in similar proportions. It is well known that pneumonic pasteurellosis in swine is often the result of a secondary infection integrated in a disease complex called “Mycoplasma-induced respiratory disease”. In this respect, sharing of ribotypes by isolates from diseased and healthy animals is not surprising. On the other hand, opportunistic infections caused by enteric bacteria, such as *Escherichia coli*, are often associated with non pathogenic commensal strains of high genetic diversity (White et al., 1990; Whittam, 1995). Our results substantiate the recent hypothesis that the natural low diversity of commensal strains occupying the nasopharynx of healthy pigs accounts for the limited genetic diversity of *P. multocida* strains associated with porcine pneumonia (Davies et al., 2003). At the same time our comparison of clinical and commensal strains questions another hypothesis, based on the possibility that the diversity of commensal strains in the nasopharynx is relatively high, implying that *P. multocida* is not a secondary pathogen but that virulent clones may have a pri-
mary role in the pathogenesis of porcine pneumonia. However, this is still an open question, as differences in the animal’s environment, such as free roaming, healthy farming or intensive housing, may have an influence on the distribution and variety of porcine strains.

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

Strains of ribotype patterns IA-7 and IIA-1 were strongly associated with capsular type A, whereas isolates of ribotype pattern IA-1 were associated with capsular type D (Table 2, Figure 2). Hence, in contrast to other authors we only rarely found mixed ribotype patterns regarding the capsular types of the strains (Davies et al., 2003). In recent publications it has been demonstrated or at least hypothesized that different subpopulations of \textit{P. multocida} may have been evolved by horizontal transfer of \textit{cap} genes and of \textit{toxA} or by a conversion of \textit{toxA}-encoding phages. In our study, this could have been happened to those strains exhibiting a virulence and capsular gene pattern typical of a distinct ribotype pattern but clustering in another. However, our data more likely indicate an independent evolution of different lineages of strains resulting in the formation of various subpopulations.

In conclusion, this study provides new knowledge on the diversity and virulence gene properties of German porcine \textit{P. multocida} strains. The association of a small number of
ribotype patterns with porcine pneumonia and PAR and the coexistence of these clinical strains with those of the normal respiratory flora of clinically healthy swine suggests a limited genetic diversity, in that, only a few dominant *P. multocida* subpopulations constitute the group of opportunistic pathogens. It is noteworthy to remember that all three major ribotypes contained strains exhibiting different virulence features, mainly regarding their capsular type and the presence or absence of *toxA* and *pfhaB*, which suggests some kind of specialization of these subpopulations probably for certain host niches or environments which could be an interesting issue for further investigations. Future vaccine strategies against porcine pasteurellosis and PAR should consider strains of the three major subpopulations to ensure protection against *P. multocida* infections in swine as best possible.

**Acknowledgements**

We greatly acknowledge the receipt of *P. multocida* reference strains from the late Prof. Dr. R.B. Rimler, National Animal Disease Center, Ames, USA. This work was financed by IDT Biologika GmbH Dessau-Roßlau, Germany.
References


Table 1: Distribution of virulence-associated genes among 382 *P. multocida* strains related to anatomic site of isolation and anamnestic information
Table 2: Origin, anamnesis, PCR and ribotyping results of 382 porcine Pasteurella multocida strains
**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.
Figure 2: Dendrogram based on *HpaII* ribotypes obtained with 382 *P. multocida* strains
Table 1: Distribution of virulence-associated genes among 382 *P. multocida* strains related to anatomic site of isolation and anamnestic information

<table>
<thead>
<tr>
<th>Number (percentage) of strains positive for the respective gene and gene combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>capA</td>
</tr>
<tr>
<td>Total number of strains</td>
</tr>
<tr>
<td>Anatomic site</td>
</tr>
<tr>
<td>URT (n=205)</td>
</tr>
<tr>
<td>LRT (n=157)</td>
</tr>
<tr>
<td>Unknown (n=20)</td>
</tr>
<tr>
<td>Disease status</td>
</tr>
<tr>
<td>Clinically healthy (n=169)</td>
</tr>
<tr>
<td>Clinically ill (n=189)</td>
</tr>
<tr>
<td>Pneumonia (n = 155)</td>
</tr>
<tr>
<td>PAR (n = 25)</td>
</tr>
<tr>
<td>Miscellaneous (n = 9)</td>
</tr>
<tr>
<td>Unknown (n=24)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>No. of strains patterns</th>
<th>No. of strains per pattern</th>
<th>Ribotype patterns</th>
<th>No. of strains positive for the respective genes</th>
<th>Anamnesis</th>
<th>Anatomic origin of samples</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>capA</td>
<td>capD</td>
<td>capF</td>
<td>cap-neg</td>
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<tr>
<td></td>
<td>Cluster</td>
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<td>Pattern</td>
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<td>I</td>
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<td>1</td>
<td>I</td>
<td>298</td>
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<tr>
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<tr>
<td>I</td>
<td>A</td>
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<td>I</td>
<td>1</td>
<td>0</td>
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<tr>
<td>I</td>
<td>A</td>
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<td>I</td>
<td>2</td>
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<td>I</td>
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</tr>
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<td>I</td>
<td>A</td>
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<td>I</td>
<td>A</td>
<td>7</td>
<td>I</td>
<td>115</td>
<td>112</td>
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<td>I</td>
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<td>I</td>
<td>B</td>
<td>83</td>
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<td>II</td>
<td>1</td>
<td>1</td>
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<tr>
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<td>A</td>
<td>3</td>
<td>II</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Σ: 382</td>
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<td></td>
<td></td>
<td>204</td>
<td>175</td>
</tr>
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</table>
*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
Supplemental Table 1: Sequence, specificity, and PCR conditions applied for the detection of virulence-associated genes in *P. multocida* strains

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5´- 3´)</th>
<th>Detected gene</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMT1 s/as</td>
<td>ATCCGCTATTTACCCAGTG/GCTGTAACGAACTCGCA</td>
<td>kmt1</td>
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<td>Townsend et al. (2001)</td>
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<tr>
<td>CapA s/as</td>
<td>TGCCAAAATCGCAGTCAG/TTGCCATCATTTGTCAG</td>
<td>hyaD/C</td>
<td>1044</td>
<td>Townsend et al. (2001)</td>
</tr>
<tr>
<td>CapB s/as</td>
<td>CATTTATCCAAGCTCCACC/GCCGAGAGTTTCAATCC</td>
<td>bcbB</td>
<td>759</td>
<td>Townsend et al. (2001)</td>
</tr>
<tr>
<td>CapD s/as</td>
<td>TTAACAAAAAGAAGACTAGA/GGACCAAGCCAACCTCAACCATCGAG</td>
<td>dcbF</td>
<td>657</td>
<td>Townsend et al. (2001)</td>
</tr>
<tr>
<td>CapE s/as</td>
<td>TCCGCAGAAAAATTTATGACTC/GCTGCTGTGATTTCGTC</td>
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<td>Townsend et al. (2001)</td>
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<tr>
<td>CapF s/as</td>
<td>AATCGGAGAAGCGAGAATCA/TTTGCGGCTCAATCTCTG</td>
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<td>Townsend et al. (2001)</td>
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<td>Fim4 s/as</td>
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<td>500</td>
<td>Doughty et al., (2000)</td>
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<td>ToxA s*/as*</td>
<td>CTTAGATGACGCAAGTGT/CAAGTGCCACACCCTCTAT</td>
<td>toxA</td>
<td>848</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>ToxA s/tonB</td>
<td>ATGAAAACAAAACATTTTATTGACTGG/GCTGCTGATGTC</td>
<td>toxA</td>
<td>3858</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>Oma87 s/as</td>
<td>ATGAAAAAACTTTTATGCGAAGTC/TTTGCTGCTGATGTC</td>
<td>oma87</td>
<td>949</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>NanB s/as</td>
<td>GTTTCTAAAGTGACGCA/ACAGCAAAGGAAAGACTGTCC</td>
<td>nanB</td>
<td>585</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>NanH s/as</td>
<td>GAATATGCCGGAACCAATCA/TTTGCGGCAACACCCTCT</td>
<td>nanH</td>
<td>361</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>ExbB s/tonB as</td>
<td>GGTGTTGATATTGATGCGG/GCAGTGCTGCTGAGGTT</td>
<td>exbBD/tonB</td>
<td>1144</td>
<td>Ewers et al. (2006)</td>
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<tr>
<td>PflaB s*/as*</td>
<td>AGCTGATCAAGTGGTGAAC/TGGTACATTGGAATGCTG</td>
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<tr>
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<td>Detected gene</td>
<td>Product size (bp)</td>
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<td>------------</td>
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</tr>
<tr>
<td>HgbA s/as</td>
<td>TGGCGGATAGTCATCAAG/CCAAAGAACCACTACCCA</td>
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<td>420</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>HgbB s*/as*</td>
<td>ACCGCGTTGGAATTATGATTG/CATTGAGTACGGCTTGACAT</td>
<td>hgbB</td>
<td>789</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>TbpA s*/as*</td>
<td>TTGGTTGAAACGGAAGGC/TAACGTGTACGGAAAAGCCC</td>
<td>tspA</td>
<td>729</td>
<td>Ewers et al. (2006)</td>
</tr>
<tr>
<td>TbpA s/as</td>
<td>TGCGACAACGGAAGACTCCTC/GGACAGTGCATATAACTCTTACTA</td>
<td>tspB</td>
<td>2073</td>
<td>This study</td>
</tr>
<tr>
<td>16SrRNA s/as</td>
<td>ATGCAAGTCGAACCGGTAGCAGGGTGACAGAGGGGC/TCATGCATACCCTGCTCGAT</td>
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<td>1355</td>
<td>This study</td>
</tr>
<tr>
<td>Hssb s/as</td>
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<td>hssB</td>
<td>1685</td>
<td>This study</td>
</tr>
</tbody>
</table>

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