Veterinary Microbiology 233 (2019) 190–195

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Original research paper

Virulence gene profiling and *ompA* sequence analysis of *Pasteurella multocida* and their correlation with host species

B. Ujvári[a], L. Makrai[b], T. Magyar[a,*]

[a] Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, P.O. Box 18, 1581 Budapest, Hungary
[b] Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, Budapest, Hungary

ARTICLE INFO

Keywords:
Pasteurella multocida
Virulence-Associated genes
ompA
Molecular typing

ABSTRACT

This study describes the prevalence of capsule biosynthesis genes, LPS genotypes, virulence associated genes and the analysis of the outer membrane protein (*ompA*) sequence of *Pasteurella multocida* isolates (n = 180) from different locations in Hungary, from various host species, including humans. When combining capsular types with LPS genotypes, eight capsule - LPS genotype combinations were detected. A: L3 was the most dominant in bovine and porcine isolates, A: L1 in feline and human isolates, while D: L3 was the most common among strains from small ruminants. The *P. multocida* toxin encoding gene *toxA* was highly prevalent among small ruminant and porcine strains, while in human, feline and bovine isolates it could not be detected. Combination of the tested virulence associated genes (*hgbA, nanH, hgbB, tbpA, pfhA, hsf1, hsf2, tadD, ptfA*) classified our *P. multocida* isolates into 13 different virulence gene profiles (VGPs). These VGPs showed an association with host species. Analysis of the *ompA* sequence data confirmed this distribution by host species, which may indicate that host adaptation is taking place. The typing scheme used in this study may be useful in epidemiological investigations.

1. Introduction

Pasteurella multocida is a Gram-negative opportunistic bacterial pathogen that causes various diseases in a wide range of host species including humans worldwide (Wilkie et al., 2012; Wilson and Ho, 2013). The organism causes fowl cholera (Rhoades and Rimler, 1989), atrophic rhinitis in pigs (Magyar and Lax, 2002), haemorrhagic septicaemia in buffalo and cattle (De Alwis, 1992), pneumonia in ruminants and swine, and respiratory tract diseases in rabbits and rodents (Boyce et al., 2010).

P. multocida is a diverse bacterial species, consisting of five capsular serogroups (Carter, 1955; Rimler and Rhoades, 1987), sixteen serovars based on the lipopolysaccharide (LPS) antigens (Heddleston et al., 1972; Harper et al., 2011) and eight LPS genotypes (Harper et al., 2015). It has a complex set of virulence associated genes (Harper et al., 2006) and several putative virulence factors have been identified that contribute to adhesion, colonization and invasion of the host. These include adhesins, the *P. multocida* toxin (PMT), iron-acquisition proteins, extracellular enzymes (neuraminidases) and outer membrane proteins (Harper et al., 2006). Molecular characterisation based on the detection of different virulence associated genes has proven to be useful for typing *P. multocida* isolates, and these virulence factors are thought to be important epidemiological markers for recognition of the pathogenic potential of the isolates (Ewers et al., 2006; Atashpaz et al., 2009; García-Alvarez et al., 2017).

For this reason, the aim of this study was to screen 180 Hungarian *P. multocida* isolates recovered from different host species (including humans) for the prevalence of capsular serovars, LPS genotypes and virulence-associated genes, and in addition the gene encoding outer membrane protein A (*ompA*), and correlate the carriage of these virulence traits to host species.

2. Materials and methods

2.1. Bacterial isolates

A total of 180 isolates of *P. multocida* isolated from different host species were used in this study (Supplementary Table 1). These strains were isolated from cattle, small ruminants (sheep and goat), pigs, cats and humans in Hungary from 1988 to 2018. The isolates were cultured on Columbia agar (LAB M Ltd., Bury, UK) plates supplemented with 5% sheep blood under aerobic conditions at 37 °C for 24 h. Their identity was confirmed by a species-specific PCR assay (Townsend et al., 1998). Combinations of oligonucleotide primers were used for amplification of *kmt1* (species identification), *toxA* (PMT) and *hyaC-hyaD* (capsular serogroup A) sequences in the same reaction (Gautam et al., 2004;

* Corresponding author.
E-mail address: magyar.tibor@agrar.mta.hu (T. Magyar).

https://doi.org/10.1016/j.vetmic.2019.05.005
Received 26 March 2019; Received in revised form 2 May 2019; Accepted 3 May 2019

Register and DeJong, 2006). The other capsular types (D and F) were identified using a multiplex PCR method as described previously (Townsend et al., 2001). LPS genotype was determined using the LPS multiplex PCR (Harper et al., 2015).

2.2. Virulence-associated genes

For DNA preparation, a loopful of cultured bacterial growth was suspended in 50 µL sterile double-distilled water and heated in a thermal cycler for 20 min at 99 °C. Cellular debris were pelleted by centrifugation and the supernatant was used as DNA template for PCR amplification. To confirm the presence of virulence-associated genes, type I and type IV fimbrial subunits (*fimA*, *ptfA*), autotransporter adhesins (*hsf-1*, *hsf-2*), tight adherence protein D (*tadD*) and filamentous haemagglutinin (*pfhA*) coding genes were detected by PCR as previously described (Sellyei et al., 2010; Tang at el., 2009). The prevalence of genes encoding iron acquisition proteins (*hgbA*, *hgbB, tbpA*) and the neuraminidase gene *nanH* was investigated according to the procedure of Ewers et al. (2006) and Atashpaz et al. (2009). All reactions were performed using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

2.3. Phylogenetic analysis of the ompA gene

The *ompA* gene sequence was analysed in a selection of 94 strains of *P. multocida* from different host species representing each geographic region, serovar and virulence gene profile (Supplementary Table 1). Amplification of the *ompA* gene was generated with primers designed to amplify an *ompA* gene segment between nucleotides 1-1077 by a PCR as described by Katoch et al. (2014). Sequencing of PCR products was performed by Macrogen Europe (Amsterdam, The Netherlands). Nucleotide sequences of the partial *ompA* gene sequences were aligned and compared using BioEdit software (version 7.2.3) (Hall, 2011). Nucleotide sequence data were analysed using MEGA7 software (Kumar et al., 2016). The evolutionary history was inferred using the neighbour-joining model with the p-distance method, and the dataset was subjected to bootstrap analysis of 1000 replicates. The GenBank accession numbers for the *ompA* sequences obtained in this study are MK805100 – MK805124.

2.4. Statistical analysis

Using the program GraphPad QuickCalcs (GraphPad Software, San Diego, CA, USA), Fisher's exact test was used to evaluate the possible associations between virulence gene profiles and *ompA* clusters, and p values of < 0.05 were considered statistically significant.

3. Results

The isolates exhibited colony morphology typical of *P. multocida*, and their identity was confirmed by the species-specific PCR assay. Capsular typing detected two capsular genotypes (A and D) for the bovine, ovine, caprine and porcine strains of *P. multocida*. The isolates from human cases were capsular serogroup A or F, and all strains from cats belonged to capsular type A. Type A was the most common capsular serovar for *P. multocida* strains from bovine, ovine, porcine, human and feline strains, while the predominant capsular type was type D in caprine isolates (Table 1).

LPS typing identified two LPS genotypes for the isolates from cattle and sheep in a very similar ratio (L3: 87.7% and 73.3%, L6: 12.3% and 26.7%, respectively) (Table 1). Only one LPS genotype (L3) was found in *P. multocida* isolates from goats and swine. In isolates from human cases, five categories of LPS genotypes (L1, L3, L4, L5 and L7) were detected. L1 (53.6%) was the dominant genotype, followed by L3 (32.1%) while L4 (7.1%), L5 (3.6%) and L7 (3.6%) occurred only in a smaller proportion. For the feline *P. multocida* isolates, L1 (72.7%), L3 (9.1%) and L7 (18.2%) LPS genotypes were found.

When capsular types were combined with LPS genotypes, the most commonly identified type was A: L3 for the isolates of bovine (77.8%), and porcine (72.5%) origin. For *P. multocida* strains from sheep and goats, D: L3 (33.3% and 80.0%) was the most common one, while A: L1 was the dominant type for isolates from humans (46.5%) and cats (72.7%) (Table 1).

The *toxA* gene was detected in all ovine isolates, and in a high proportion of caprine and porcine strains (40.0% and 55.0%), but was not present in isolates from humans, cats and cattle. Among the caprine *P. multocida* strains, *toxA* gene was found only in pneumonic, capsular type D isolates. In porcine pneumonic isolates, we found a lower prevalence of the *toxA* gene (38.8%) as compared to strains recovered from nasal swabs (68.2%). In relation to the capsular type, we found a higher prevalence of *toxA* in capsular type A strains than in capsular type D isolates, both in porcine pneumonic (40.0%) and nasal swab (71.4%) isolates (Table 2).

Type I fimbrial subunit encoding gene *fimA* was found in all *P. multocida* strains included in the study, therefore it was not analysed further. Based on the presence or absence of the other nine virulence-associated genes, we distinguished 13 different virulence-associated gene profiles (VGP) (Table 3). With a few exceptions, each VGP was associated with only one host species. Bovine *P. multocida* isolates were exclusively grouped into VGP 1, 2, or 3, with the dominance of VGP 1 (61.7%). The ovine strains were assigned to VGP 4 and 5, of which VGP 5 was the more common (67%), and caprine isolates were grouped into VGP 5. Porcine *P. multocida* strains were assigned to four VGPs (6–9). The majority of these strains belonged to VGP 9 (70%). *P. multocida* isolates from cats were assigned into VGP 12 (82%) and 13 (18%). *P. multocida* strains from humans were more diverse, they belonged to six VGPs (6, 9–13), of which VGP 12 (60.7%) was the dominant type. (Table 4).

The *ompA* genes of the 94 *P. multocida* strains examined represented 25 unique, but closely relatedsequence types (Fig. 1a) forming 9 clusters of allelic variants (Cluster A–I). Cluster A was the most prevalent *ompA*-type (35.1%), followed by Cluster G (17.0%), Cluster D (13.8%) and Cluster I (11.7%). The remaining *ompA* clusters were less common (Fig. 1, Supplementary Fig. 1, Supplementary Table 1). *OmpA* clusters correlated with VGPs. Cluster A was associated with VGP 1 and 5, representing *P. multocida* isolates from cattle and small ruminants, respectively. *OmpA* cluster B was exclusively associated with VGP 3 (bovine isolates). *OmpA* cluster C enclosed VGP 6 and 7, including porcine strains with one human isolate. Cluster D was related to VGP 2 (bovine strains) and VGP 10 (one human isolate). Cluster E included the porcine VGP 8 strains, while cluster F was associated with human VGP 12 isolates. *OmpA* cluster G contained VGP 9 strains of porcine and human origin. *OmpA* cluster H included ovine strains (VGP 4) and a single human isolate (VGP 11). *OmpA* cluster I was build up by VGP 12 and 13 isolates from cats and humans (Supplementary Fig. 1a). Virulence gene profiles and *ompA* clusters described above were significantly associated ($p < 0.05$). Analysis of the *ompA* proteins encoded by the *ompA* gene sequences in this study resulted a phylogenetic tree containing identical *ompA* clusters as the tree based on nucleotide sequences (Supplementary Fig. 1b).

4. Discussion

P. multocida is a widespread veterinary pathogen of mammals and birds with zoonotic potential (Wilson and Ho, 2013). In the present study, 180 isolates of *P. multocida* from a broad range of host species were characterised comprehensively by determining their capsular type, LPS genotype. In addition, a virulence gene profile (VGP) scheme has been established analysing nine virulence-associated genes. Furthermore, *ompA* sequence analysis of 94 selected strains revealed an association between host species, VGP and the *ompA* sequence type of the examined *P. multocida* strains.

Table 1
Percent distribution of different capsular type: LPS genotype combinations in *P. multocida* isolated from different host species. The number of isolates is displayed in parentheses.

Capsule: LPS genotype	Cattle (n = 81)	Sheep (n = 15)	Goat (n = 5)	Swine (n = 40)	Human (n = 28)	Cat (n = 11)
A: L1	0	0	0	0	46.5 (13)	72.7 (8)
A: L3	77.8 (63)	40.0 (6)	20.0 (1)	72.5 (29)	32.1 (9)	9.1 (1)
A: L4	0	0	0	0	7.1 (2)	0
A: L5	0	0	0	0	3.6 (1)	0
A: L6	12.3 (10)	26.7 (4)	0	0	0	0
A: L7	0	0	0	0	3.6 (1)	18.2 (2)
D: L3	9.9 (8)	33.3 (5)	80.0 (4)	27.5 (11)	0	0
F: L1	0	0	0	0	7.1 (2)	0

Table 2
Distribution of *P. multocida* toxin (PMT) producing strains according to host species, sources and capsular types.

Host species	Source	Capsular type	Number of isolates	PMT % (n)
goat	lung	A	0	0
		D	3	66.7 (2)
	nasal swab	A	1	0
		D	1	0
sheep	lung	A	8	100 (8)
		D	4	100 (4)
	nasal swab	A	2	100 (2)
		D	1	100 (1)
swine	lung	A	15	40.0 (6)
		D	3	33.3 (1)
	nasal swab	A	14	71.4 (10)
		D	8	62.5 (5)

In total, eight capsule - LPS genotype combinations were detected in our isolates, with A: L3 the most common one that occurred in strains from nearly all host species, except for caprine strains. This finding is in general agreement with previous studies which reported type A to be the capsular serogroup most often identified in isolates from cattle and swine (Davies et al., 2003; García et al., 2011; Katsuda et al., 2013; Jamali et al., 2014; Peng et al., 2018a). Others have also reported the L3 LPS genotype to occur in similarly high percentages as in the present study in isolates from cattle (Taylor et al., 2010; Peng et al., 2018a) and pigs (Lariviere et al., 1992; Jamaludin et al., 2005). However, in recent publications the increasing incidence of the L6 LPS genotype among strains from pigs is also mentioned (Peng et al., 2018b; Turni et al., 2018). In ovine and caprine isolates, we found D: L3 and A: L3 to be the most common capsule: LPS genotype combinations. Previous studies also found capsular type A and D in these host species (Ewers et al., 2006; Vougidou et al., 2015). However these other studies did not report the relatively high occurrence of capsular type D that we identified here. This result also agrees with data in the literature suggesting that capsule: LPS genotype combination A: L1 is predominant among *P. multocida* isolates from humans and cats (Ewers et al., 2006; Ujvári et al., 2019).

PMT is responsible for the characteristic pathological lesions of progressive atrophic rhinitis in swine (Magyar and Lax, 2002). Therefore, the high incidence (55.0%) of PMT among the isolates from swine was not surprising. Toxigenic strains were isolated in a larger proportion from nasal swabs (68.2%), however, a remarkable proportion of *toxA*-positive isolates could be identified in cases of pneumonia (38.8%). Nevertheless, compared to previous studies, we detected *toxA* in a higher proportion in capsular type A than capsular type D isolates (Lariviere et al., 1992; Davies et al., 2003; Bethe et al., 2009), indicating an increasing tendency of PMT production among type A strains, at least in Hungary. Furthermore, gene *toxA* was also detected in high percentages in ovine and caprine (100% and 40.0%, respectively) *P. multocida* isolates. This finding accords with the growing body of evidence showing a frequent occurrence of PMT in *P. multocida* isolates from sheep and goats (Ewers et al., 2006; Shayegh et al., 2009; García-Alvarez et al., 2017) although a 100 percent incidence has not been previously reported in sheep. Moreover, the role of PMT in small ruminants, contrary to swine, has not been established yet, and further efforts are needed to explore the potentially pathological effect of toxin production in *P. multocida* infections in sheep and goat. Furthermore our data strongly suggest that isolates from small ruminants form a distinct group within strains isolated from different mammalian host species. This view is supported by the high proportion of PMT producing strains, the exclusive presence of the *ptfA* A allele, and the fact that they belong to unique VGP groups. The affected sheep and goat farms represented various geographical locations, and no epidemiological relationship could be established among them. It is currently unclear what could have led to the emergence of remarkable differences in strains found in sheep and goats kept under extensive housing conditions without noteworthy exchange of animals between herds.

Studying virulence associated genes is important in order to be able to understand the pathogenicity of *P. multocida*, and it also seems to be

Table 3
Virulence gene profiles (VGPs) of *P. multocida* strains based on the presence of virulence associated genes.

Virulence gene profile	Host species	*hgbA*	*nanH*	*hgbB*	*tbpA*	*pfhA*	*hsf1*	*hsf2*	*tadD*	*ptfA**
VGP 1	cattle	+	+	–	+	+	–	+	+	B
VGP 2	cattle	+	+	–	+	–	–	+	+	B
VGP 3	cattle	+	+	–	+	–	–	+	–	B
VGP 4	sheep, goat	+	+	–	+	–	–	+	–	A
VGP 5	sheep, goat	+	–	–	+	–	–	–	–	A
VGP 6	swine, human	+	+	–	–	+	–	+	+	B
VGP 7	swine	+	+	–	–	–	–	+	+	B
VGP 8	swine	+	+	+	–	–	–	+	+	B
VGP 9	swine, human	+	+	+	–	–	+	+	–	B
VGP 10	human	+	+	–	–	+	+	+	–	B
VGP 11	human	+	–	+	–	–	+	+	–	B
VGP 12	human, cat	+	+	+	–	–	–	+	–	B
VGP 13	human, cat	–	+	+	–	–	–	+	–	B

* *ptfA* allelic variant.

Table 4
Percent distribution of virulence gene profiles in *P. multocida* isolated from different host species. The number of isolates is displayed in parentheses.

Virulence gene profile	Cattle (n = 81)	Sheep (n = 15)	Goat (n = 5)	Swine (n = 40)	Human (n = 28)	Cat (n = 11)
VGP 1	61.7 (50)	0	0	0	0	0
VGP 2	28.4 (23)	0	0	0	0	0
VGP 3	9.9 (8)	0	0	0	0	0
VGP 4	0	33.3 (5)	0	0	0	0
VGP 5	0	66.7 (10)	100 (5)	0	0	0
VGP 6	0	0	0	17.5 (7)	3.6 (1)	0
VGP 7	0	0	0	5.0 (2)	0	0
VGP 8	0	0	0	7.5 (3)	0	0
VGP 9	0	0	0	70.0 (28)	17.8 (5)	0
VGP 10	0	0	0	0	3.6 (1)	0
VGP 11	0	0	0	0	3.6 (1)	0
VGP 12	0	0	0	0	60.7 (17)	81.8 (9)
VGP 13	0	0	0	0	10.7 (3)	18.2 (2)

a promising tool for characterisation of isolates. Using analysis based on virulence associated genes, we could identify several different virulence gene profiles (VGP) among *P. multocida* strains tested in this study. However, it has been challenging to compare the results of this work with those of previous studies because they investigated different panels of virulence associated genes. In general, our results and the previous reports have equally indicated that *P. multocida* isolates carry diverse sets of virulence genes that may be characteristic to the host species.

We assigned all strains isolated from ruminants into one of the *tbpA* positive virulence gene profiles (VGP 1-5). Ewers et al. (2006) found a slightly lower prevalence of this gene in *P. multocida* from cattle and sheep (70.2% and 80.0%, respectively) than we identified, which might be due to diversity of isolates from various countries. Other studies have also reported high prevalence of the *tbpA* gene (Shayegh et al., 2009; Katsuda et al., 2013; Verma et al., 2013), and in agreement with our data, *tbpA* has not been identified in isolates from non-ruminant host species (Ewers et al., 2006; García et al., 2011; Ferreira et al., 2015; Ujvári et al., 2019).

The *pfhA* and *tadD* genes were detected in the majority of bovine isolates and in a smaller proportion of porcine strains. In previous studies (Ewers et al., 2006; Tang et al., 2009; García et al., 2011; Verma et al., 2013), *pfhA* and *tadD* have also been associated with these host species, suggesting that these genes may play a role in the adherence to both bovine and swine epithelial cells.

Consistent with previous observations (Tang et al., 2009; Ferreira et al., 2015; Ujvári et al., 2019), autotransporter adhesin *hsf2* was detected in the majority of *P. multocida* isolates, regardless of host species, while *hsf1* was identified only in a high percentage in porcine (70.0%) and to a lesser extent in human (7.2%) isolates.

Type I and IV fimbrial subunit genes (*fimA* and *ptfA*) are among the sequences encoding the major surface components in *P. multocida* (Ruffolo et al., 1997; Harper et al., 2006), and they were detected in all strains tested in our study. Concerning *ptfA,* we identified both type A and B allelic variants of this gene. Interestingly, the *ptfA* allelic variant A was found only in *P. multocida* isolates from small ruminants, while the strains from all other examined host species were of type B. Sellyei et al. (2010) found that strains from birds with acute fowl cholera presented as *ptf*A type A, while strains from cases of chronic fowl cholera were classified as *ptf*A type B. It is unclear whether the presence of *ptf*A type A in *P. multocida* isolates from small ruminants has any epidemiological relevance.

The majority of *P. multocida* strains included in our study harboured the neuraminidase coding gene *nanH*, except for a small number of isolates from humans and small ruminants, and the frequency of isolation was similar to those reported previously (Ewers et al., 2006; Tang et al., 2009; Verma et al., 2013).

Regardless of host species, the majority of *P. multocida* strains carried *hgbA*, except for some human and feline isolates. The widespread prevalence of *hgbA* gene has also been established in previous publications (Bosch et al., 2002; Ewers et al., 2006; Aski and Tabatabaei, 2016). On the other hand, the *hgbB* gene showed different ratios of incidence depending on the host species. In isolates from cattle and small ruminants, *hgbB* was not detected, while it could be identified in

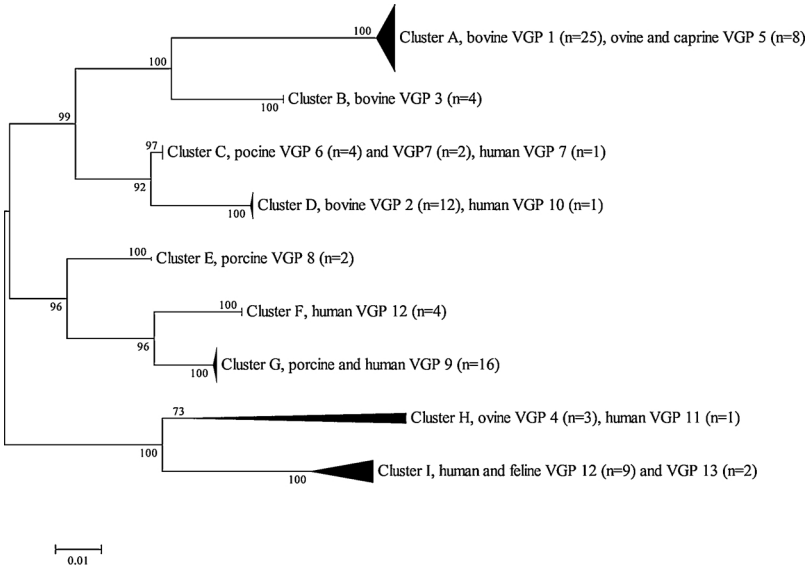

Fig. 1. Collapsed phylogenetic tree of partial *ompA* genes. The branches of the tree are indicated by the prevalence of their representatives, and are shown as triangles or vertical lines. Host species, *ompA* cluster, virulence gene profiles (VGPs) and the number of isolates are displayed next to the branches. The evolutionary history was inferred using the neighbour-joining method. Evolutionary analyses were conducted in MEGA7 software (Kumar et al., 2016).

high percentages in strains of porcine, human and feline origin. Previous publications have also reported a lower incidence of *hgbB* in *P. multocida* strains from cattle, although in small ruminants they detected it in nearly all isolates (Ewers et al., 2006; Aski and Tabatabaei, 2016).

The comparative analysis of the VGPs of our *P. multocida* isolates confirmed the diversity of this bacterial species, revealing the existence of at least 13 different profiles, and these groups showed a remarkable association with the host species. On the other hand, human isolates were an exception to the rule being present in six VGPs. In two of these groups, only human isolates were found, while two of the other groups also contained isolates from swine and two other groups also contained isolates from cats. The most likely explanation for this is that man is not a natural host for *P. multocida*, and thus humans presumably acquire the infection through contact with household pets or commercial farm animals. Accordingly, recent studies also drew attention to the existence of shared genotypes of *P. multocida* across humans and various animal species (Turni et al., 2018; Ujvári et al., 2019). Although two VGP groups only contained human *P. multocida* isolates, these types only occurred in individual cases, and it is possible that their animal-related counterparts could be identified by testing additional animal-derived strains. In previous years, mainly individual cases were reported, and only a limited number of comparative studies on human *P. multocida* strains were performed. Marois et al. (2009) characterised porcine and human *P. multocida* isolates from France using pulse-field gel-electrophoresis, concluding that strains from humans were genetically more diverse than those of porcine origin.

Recent publications (Harper et al., 2006; Katoch et al., 2014) reported that *ompA* gene variability had an effect on the virulence of *P. multocida*. The current analysis of sequence data revealed that most of the polymorphic sites of the *ompA* gene were identified in the four hypervariable extracellular loops of the transmembrane domains, and the rest of the protein amino acid sequence revealed a high degree of conservation. A more detailed examination of the domain structures showed that insertions/deletions and non-synonymous substitutions were present outside the transmembrane domains, but within the transmembrane areas, we could identify synonymous substitutions. It was generally concluded, that the structure of the *ompA* protein was preserved (data not shown). The present study discovered differences in *ompA* sequence types of *P. multocida* in association with host species and virulence gene profile, which may indicate host preference and clonality among *P. multocida* isolates. *OmpA* is one of the major outer membrane proteins of *P. multocida* (Harper et al., 2006). It thus is exposed to continuous environmental changes, whereby it undergoes strong selective pressure, leading to the development of variation in its structure. However, compared to *ompA* clusters, virulence gene profiling was able to distinguish more groups, and *ompA* clusters were not such a good indicator of host preference as VGPs. This may be because VGPs have been determined based on a combination of multiple virulence associated genes, while *ompA* sequence analysis created clusters using a single gene. Based on our findings, VGP determination together with *ompA* sequence analysis is a valuable tool for the investigation of the diversity and host preference of *P. multocida*.

Multilocus sequence typing (MLST) has widely been applied for analysing *P. multocida*. Hotchkiss et al. (2011) characterised isolates from different host species, mainly from bovine respiratory and non-respiratory cases. With the use of the RIRDC MLST scheme, they differentiated *P. multocida* isolates within and between host species, and was able to identify isolates associated strongly, but not exclusively to the bovine respiratory tract niche. In addition, they also detected sequence types (STs) shared across a wide range of host species, including isolates of avian, bovine, porcine and human origin. Recently, a comparative study on human *P. multocida* isolates was performed by our research team (Ujvári et al., 2019) that included both the assessment of potential virulence factors and population structure (MLST). A comparison of our MLST data to those of isolates in the RIRDC MLST database from a wide range of host species suggested that *P. multocida* isolates formed two distinct clusters according to the subspecies, but regardless of the host. Furthermore, Davies et al. (2004) found that outer membrane protein (OMP) profiling has more discriminatory power than MLST, and different OMP-types are associated with different genetic backgrounds. Thereby OMP-types seems to be excellent markers of clonality, and combined with data of the presence of colonisation and iron acquisition factors probably give a more accurate picture of host species adaptation of *P. multocida* than MLST.

5. Conclusion

Bacterial virulence is determined by genetic markers (Harper et al., 2006), that include colonisation factors, outer membrane proteins and iron acquisition factors. The typing scheme we have described in this study is likely to be useful for epidemiological studies. Interestingly our results demonstrate a high level of diversity in virulence gene content among *P. multocida* strains from different host species, which is likely to be important in determining differences in bacterial gene expression linked to pathology in different hosts.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence the content of the paper.

Acknowledgements

This work was supported by the National Research, Development and Innovation Fund, Hungary – Grant NKFIH K124457. The authors thank Dr. A. J. Lax for the useful comments on the manuscript. The outstanding technical assistance of É. Hegedűs and K. Oryszcsák is highly appreciated.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.005.

References

Aski, H.S., Tabatabaei, M., 2016. Occurrence of virulence-associated genes in Pasteurella multocida isolates obtained from different hosts. Microb. Pathog. 96, 52–57.
Atashpaz, S., Shayegh, J., Hejazi, M.S., 2009. Rapid virulence typing of Pasteurella multocida by multiplex PCR. Res. Vet. Sci. 87, 355–357.
Bethe, A., Wieler, L.H., Selbitz, H.J., Ewers, C., 2009. Genetic diversity of porcine Pasteurella multocida strains from the respiratory tract of healthy and diseased swine. Vet. Microbiol. 139, 97–105.
Bosch, M., Garrido, M.E., Llagostera, M., de Rozas, A.M.P., Badiola, I., Barbé, J., 2002. Characterization of the Pasteurella multocida hgbA gene encoding a hemoglobin-binding protein. Infect. Immun. 70, 5955–5964.
Boyce, J.D., Harper, M., Wilkie, I.W., Adler, B., 2010. Pasteurella. In: Gyles, C.L., Prescott, J.F., Songer, J.G., Thoen, C.O. (Eds.), Pathogenesis of Bacterial Infections of Animals, 4th ed. Blackwell Publishing, Ames, IA, pp. 325–346.
Carter, G.R., 1955. Studies on Pasteurella multocida. I. A hemagglutination test for the identification of serological types. Am. J. Vet. Res. 16, 481–484.
Davies, R.L., MacCorquodale, R., Baillie, S., Caffrey, B., 2003. Characterization and comparison of Pasteurella multocida strains associated with porcine pneumonia and atrophic rhinitis. J. Med. Microbiol. 52, 59–67.
Davies, R.L., MacCorquodale, R., Reilly, S., 2004. Characterisation of bovine strains of Pasteurella multocida and comparison with isolates of avian, ovine and porcine origin. Vet. Microbiol. 99, 145–158.
De Alwis, M.C.L., 1992. Haemorrhagic septicaemia—a general review. Br. Vet. J. 148, 99–112.
Ewers, C., Lübke-Becker, A., Bethe, A., Kießling, S., Filter, M., Wieler, L.H., 2006. Virulence genotype of Pasteurella multocida strains isolated from different hosts with various disease status. Vet. Microbiol. 114, 304–317.
Ferreira, J., Treger, K., Busey, K., 2015. Pneumonia and disseminated bacteremia with Pasteurella multocida in the immune competent host: a case report and a review of the literature. Respir. Med. Case Rep. 15, 54–56.
García, N., Fernández-Garayzábal, J.F., Goyache, J., Domínguez, L., Vela, A.I., 2011. Associations between biovar and virulence factor genes in Pasteurella multocida isolates from pigs in Spain. Vet. Rec. 169 362-362.
García-Alvarez, A., Vela, A.I., San Martín, E., Chaves, F., Fernández-Garayzábal, J.F.,

Lucas, D., Cid, D., 2017. Characterization of Pasteurella multocida associated with ovine pneumonia using multi-locus sequence typing (MLST) and virulence-associated gene profile analysis and comparison with porcine isolates. Vet. Microbiol. 204, 180–187.
Gautam, R., Kumar, A.A., Singh, V.P., Singh, V.P., Dutta, T.K., Shivachandra, S.B., 2004. Specific identification of Pasteurella multocida serogroup-A isolates by PCR assay. Res. Vet. Sci. 76, 179–185.
Hall, T., 2011. BioEdit: an important software for molecular biology. GERF Bull Biosci 2, 60–61.
Harper, M., Boyce, J.D., Adler, B., 2006. Pasteurella multocida pathogenesis: 125 years after Pasteur. FEMS Microbiol. Lett. 265, 1–10.
Harper, M., Cox, A.D., Adler, B., Boyce, J.D., 2011. Pasteurella multocida lipopolysaccharide: The long and the short of it. Vet. Microbiol. 153, 109–115.
Harper, M., John, M., Turni, C., Edmunds, M., St Michael, F., Adler, B., Blackall, P.J., Cox, A.D., Boyce, J.D., 2015. Development of a rapid multiplex PCR to genotype Pasteurella multocida strains using the lipopolysaccharide outer core biosynthesis locus. J. Clin. Microbiol. 53, 477–485.
Heddleston, K.L., Gallagher, J.E., Rebers, P.A., 1972. Fowl cholera: gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. Avian Dis. 16, 925–936.
Hotchkiss, E.J., Hodgson, J.C., Lainson, F.A., Zadoks, R.N., 2011. Multilocus sequence typing of a global collection of Pasteurella multocida isolates from cattle and other host species demonstrates niche association. BMC Microbiol. 11, 115.
Jamali, H., Rezagholipour, M., Fallah, S., Dadrasnia, A., Chelliah, S., Velappan, R.D., Wei, K.S.C., Ismail, S., 2014. Prevalence, characterization and antibiotic resistance of Pasteurella multocida isolated from bovine respiratory infection. . Vet. J 202, 381–383.
Jamaludin, R., Blackall, P.J., Hansen, M.F., Humphrey, S., Styles, M., 2005. Phenotypic and genotypic characterisation of Pasteurella multocida isolated from pigs at slaughter in New Zealand. New Zeal. Vet. J 53, 203–207.
Katoch, S., Sharma, M., Patil, R.D., Kumar, S., Verma, S., 2014. In vitro and in vivo pathogenicity studies of Pasteurella multocida strains harbouring different ompA. Vet. Res. Commun. 38, 183–191.
Katsuda, K., Hoshinoo, K., Ueno, Y., Kohmoto, M., Mikami, O., 2013. Virulence genes and antimicrobial susceptibility in Pasteurella multocida isolates from calves. Vet. Microbiol. 167, 737–741.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Lariviere, S., Leblanc, L., Mittal, K.R., Martineau, G.P., 1992. Characterization of Pasteurella multocida from nasal cavities of piglets from farms with or without atrophic rhinitis. J. Clin. Microbiol. 30, 1398–1401.
Magyar, T., Lax, A.J., 2002. Atrophic rhinitis. In: Brogden, K.A., Guthmiller, J.M. (Eds.), Polymicrobial Diseases. ASM Press, Washington DC, pp. 169–197.
Marois, C., Fablet, C., Gaillot, O., Morvan, H., Madec, F., Kobisch, M., 2009. Molecular diversity of porcine and human isolates of Pasteurella multocida. J. Appl. Microbiol. 107, 1830–1836.
Peng, Z., Wang, H., Liang, W., Chen, Y., Tang, X., Chen, H., Wu, B., 2018a. A capsule/lipopolysaccharide/MLST genotype D/L6/ST11 of Pasteurella multocida is likely to be strongly associated with swine respiratory disease in China. Arch. Microbiol. 200, 107–118.
Peng, Z., Liang, W., Wang, F., Xu, Z., Xie, Z., Lian, Z., Hua, L., Zhou, R., Chen, H., Wu, B., 2018b. Genetic and phylogenetic characteristics of Pasteurella multocida isolates from different host species. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.01408.
Register, K.B., DeJong, K.D., 2006. Analytical verification of a multiplex PCR for identification of Bordetella bronchiseptica and Pasteurella multocida from swine. Vet. Microbiol. 117, 201–210.
Rhoades, K.R., Rimler, R.B., 1989. Fowl cholera. In: Adlam, C.F., Rutter, J.M. (Eds.), Pasteurella and Pasteurellosis. Academic Press, London, pp. 95–113.
Rimler, R.B., Rhoades, K.R., 1987. Serogroup F, a new capsule serogroup of Pasteurella multocida. J. Clin. Microbiol. 25, 615–618.
Ruffolo, C.G., Tennent, J.M., Michalski, W.P., Adler, B., 1997. Identification, purification, and characterization of the type 4 fimbriae of Pasteurella multocida. Infect. Immun. 65, 339–343.
Sellyei, B., Bányai, K., Magyar, T., 2010. Characterization of the ptfA gene of avian Pasteurella multocida strains by allele-specific polymerase chain reaction. J. Vet. Diagn. Invest. 22, 607–610.
Shayegh, J., Sharaf, J., Mikaili, P., Namvar, H., 2009. Pheno-and genotyping of Pasteurella multocida isolated from goat in Iran. Afr. J. Biotechnol. 8, 3707–3710.
Tang, X., Zhao, Z., Hu, J., Wu, B., Cai, X., He, Q., Chen, H., 2009. Isolation, antimicrobial resistance, and virulence genes of Pasteurella multocida strains from swine in China. . J. Clin. Microbiol. 47, 951–958.
Taylor, J.D., Fulton, R.W., Dabo, S.M., Lehenbauer, T.W., Confer, A.W., 2010. Comparison of genotypic and phenotypic characterization methods for Pasteurella multocida isolates from fatal cases of bovine respiratory disease. J. Vet. Diagn. Invest. 22, 366–375.
Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M., Dawkins, H.J., 1998. Development of PCR assays for species-and type-specific identification of Pasteurella multocida isolates. J. Clin. Microbiol. 36, 1096–1100.
Townsend, K.M., Boyce, J.D., Chung, J.Y., Frost, A.J., Adler, B., 2001. Genetic organization of Pasteurella multocida cap loci and development of a multiplex capsular PCR typing system. J. Clin. Microbiol. 39, 924–929.
Turni, C., Singh, R., Blackall, P.J., 2018. Genotypic diversity of Pasteurella multocida isolates from pigs and poultry in Australia. Aust. Vet. J. 96, 390–394.
Ujvári, B., Weiczner, R., Deim, Z., Terhes, G., Urbán, E., Tóth, A.R., Magyar, T., 2019. Characterization of Pasteurella multocida strains isolated from human infections. Comp. Immunol. Microbiol. Infect. Dis. 63, 37–43.
Verma, S., Sharma, M., Katoch, S., Verma, L., Kumar, S., Dogra, V., Chahota, R., Dhar, P., Singh, G., 2013. Profiling of virulence associated genes of Pasteurella multocida isolated from cattle. Vet. Res. Commun. 37, 83–89.
Vougidou, C., Sandalakis, V., Psaroulaki, A., Siarkou, V., Petridou, E., Ekateriniadou, L., 2015. Distribution of the ompA-types among ruminant and swine pneumonic strains of Pasteurella multocida exhibiting various cap-locus and toxA patterns. Microbiol. Res. (Pavia). 174, 1–8.
Wilkie, I.W., Harper, M., Boyce, J.D., Adler, B., 2012. Pasteurella multocida: disease and pathogenesis. Curr. Top. Microbiol. Immunol. 361, 1–22.
Wilson, B.A., Ho, M., 2013. Pasteurella multocida: from zoonosis to cellular microbiology. Clin. Microbiol. Rev. 26, 631–655.