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Characterization of *Pasteurella multocida* strains isolated from human infections

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ABSTRACT

Isolates of *Pasteurella multocida* recovered from infected humans (n = 15) were characterized by traditional and molecular microbiological methods and were compared with cat-derived strains (n = 5). The most prevalent subspecies among strains from human infections was *P. multocida* subsp. *septica* (80%), and nearly all isolates showed a similar combination of virulence-associated genes. MLST analysis classified the 20 *P. multocida* strains into 16 different sequence types, and we assigned 11 new sequence types (ST), however, only one of those (ST 334) was shared by two human and one cat isolates. *P. multocida* subsp. *septica* strains formed a distinct phylogenetic group within the species. The strains showed resistance to erythromycin, clindamycin and sulfamethoxazole, and with two exceptions, resistance to tilmicosin was also detected. Each strain was susceptible to ampicillin, streptomycin, gentamycin, tetracycline, doxycycline, cefazolin, cefpodoxime, chloramphenicol, florfenicol and enrofloxacin. Common characteristics (virulence profile and antibiotic sensitivity pattern) shared by strains isolated from humans and cats support the view that domestic cats may serve as a potential reservoir for *P. multocida*.

1. Introduction

Pasteurella multocida is a widespread veterinary pathogen and it also has the potential to cause zoonotic infections in humans [1]. In animals, *P. multocida* is associated with acute to chronic diseases such as fowl cholera, atrophic rhinitis of swine, haemorrhagic septicaemia in buffalo and cattle, and respiratory diseases in various host species [2–5]. Furthermore, it is a natural inhabitant of the oropharyngeal flora of dogs and cats [6]. In humans, *P. multocida* infections have been reported during the last few decades [7–9]. Humans acquire *Pasteurella* infection primarily from injuries caused by companion animals, most typically through animal bites, scratches, or licks on skin abrasions [7,8]. Bite wound infection is the most common clinical manifestation that may result in joint infections, abscess formation, cellulitis, lymphangitis, meningitis, endocarditis, peritonitis, pneumonia or sepsis [10,11]. Systemic infections mainly affect immunocompromised individuals [12]. Although *Pasteurella canis* and *Pasteurella dagmatis* may also be present in animal bites, *P. multocida* is the most prevalent species of the *Pasteurella* genus causing human infections [13].

P. multocida strains can be classified into five capsular serogroups (A, B, D, E and F) and 16 Heddleston serovars (1–16) based on their capsular structure and lipopolysaccharide antigens [14–16]. Furthermore, 13 biovars can be differentiated according to their fermentation of different carbohydrates [17,18]. LPS structures of the 16 Heddleston type strains were extensively studied by Harper et al. [19] who described eight distinct LPS outer core biosynthesis loci (L1 – L8), and developed a multiplex PCR scheme to differentiate these genotypes.

Several molecular typing methods have been used to reveal suspected epidemiologic relationship between *P. multocida* infections in humans and contact with animals.

In a number of studies, pulsed-field gel electrophoresis (PFGE) has been used to provide evidence for the zoonotic transmission of *P. multocida* from animals to humans [10,20]. A multilocus sequence typing (MLST) scheme has also been developed for studying the genetic diversity of *P. multocida* [21] that has been used to characterize isolates from various animal species [22,23].

The majority of the studies on *P. multocida* infections in humans describe individual cases, and insufficient information is usually

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Table 1
Description and characteristics of *P. multocida* isolates obtained from humans and cats.

ID	Specimen	ST	Capsular type	Heddleston serovar	LPS PCR	*hgbA*	*hgbB*	*nanH*	*pfhA*	*hsf1*	*tadD*	Subspecies[a]	Biovar	Sorbitol fermentation
Human isolates														
Pm242	abscess	330	A	1	L1	+	+	+	–	+	–	S	2	+
Pm243	wound	276	A	3	L3	+	+	+	–	–	–	S	2	+
Pm245	abscess	332	A	1	L1	+	+	+	–	–	–	S	7	–
Pm246	abscess	333	A	1	L1	+	+	+	–	–	–	S	7	–
Pm248	abscess	334	A	1	L1	+	+	+	–	–	–	S	7	–
Pm250	wound	335	F	1	L1	+	+	+	–	–	–	S	3	+
Pm251	wound	334	A	1	L1	+	+	+	–	–	–	S	7	–
Pm252	wound	336	A	3	L3	+	+	+	–	+	–	S	3	+
Pm254	surgery	337	A	1	L1	+	+	+	–	–	–	S	7	–
3080	wound	343	A	6	L4	+	+	+	–	–	–	S	10	–
3081	wound	333	A	1	L1	+	+	+	–	–	–	S	2	+
3083	wound	336	A	3	L3	+	+	+	–	–	–	S	3	+
Pm244	trachea	331	A	3	L3	+	–	+	+	+	–	M/G	6	–
Pm247	eye surgery	13	A	3	L3	+	–	+	+	–	+	M/G	9	+
Pm249	wound	71	A	3	L3	+	+	–	–	+	–	M/G	3	+
Cat isolates														
3694	oral cavity	247	A	1	L1	+	+	+	–	–	–	S	2	+
3695	oral cavity	340	A	1	L1	+	+	+	–	–	–	S	2	+
3696	oral cavity	341	A	3	L3	+	+	+	–	–	–	M/G	3	+
3697	oral cavity	334	A	1	L1	+	+	+	–	–	–	S	7	–
3698	oral cavity	214	A	8	L7	–	+	+	–	–	–	M/G	12	+

Additional information: *toxA*, *tbpA* – all strains negative, *fimA*, *hsf2* – all strains positive, *ptfA* – all strains positive harbouring allelic variant B.
[a] S, subsp. *septica*; M/G, subsp. *multocida/gallicida*.

accumulated on the strains isolated from such cases. To date, no study has published an MLST investigation of human isolates of *P. multocida*, and only limited data are available on the virulence gene profile of *P. multocida* isolated from humans [24]. Here we report the detailed investigation of 15 *P. multocida* clinical isolates obtained from human infections in Hungary in comparison with five cat-derived strains of *P. multocida*.

2. Material and methods

2.1. Bacterial isolates and identification

The bacterial strains used in this study are described in Table 1. Fifteen *P. multocida* strains of human origin were isolated from patients at the Szent-Györgyi Albert Medical and Pharmaceutical Centre, University of Szeged, Hungary. Five strains of feline origin were used for comparison. They were isolated from the oral cavities of cats and represented various geographic locations without any connection to the human cases. 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 [25]. Combinations of oligonucleotide primers were used for amplification of *kmt1* (species identification), *toxA* (*P. multocida* toxin), and *hyaC-hyaD* (capsular serogroup A) sequences in the same reaction [26,27]. The other capsular types (B, D, E and F) were identified using a multiplex PCR method as described previously [28]. The Heddleston serovar was determined using the gel diffusion precipitin test [15], and the result was confirmed by the LPS multiplex PCR [19]. Biovars were defined via the carbohydrate fermentation patterns and ornithine decarboxylase activity determined according to the scheme developed by Fegan et al. [17] and Blackall et al. [18].

2.2. Molecular examinations

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 the DNA template for PCR amplification.

The main phylogenetic lineages of *P. multocida* were determined using PCR-RFLP (PCR followed by a restriction fragment length polymorphism assay) based on 16S rDNA polymorphisms according to the method of Sellyei et al. [29]. PCR products were digested using the FastDigest™ *Hin*dIII restriction endonuclease as per the manufacturer's instructions (Thermo Scientific). Fragment lengths were determined using agarose gel electrophoresis and images were recorded using Kodak Molecular Imaging Software (version 5.0).

To determine the presence of virulence-associated genes, multiple adhesins were tested including the type I and type IV fimbrial subunits (*fimA*, *ptfA*), autotransporter adhesins (*hsf-1*, *hsf-2*), tight adherence protein D (*tadD*) and filamentous haemagglutinin (*pfhA*). Genes were detected by PCR as previously described [30,31]. The prevalence of iron acquisition proteins (*hgbA*, *hgbB, tbpA*) and the neuraminidase gene *nanH* was investigated according to the procedure of Ewers et al. [24] and Atashpaz et al. [32]. All reactions were performed using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

MLST was performed as described by Subaaharan et al. [21]. Sequencing of PCR products was performed by Macrogen Europe (Amsterdam, The Netherlands). Nucleotide sequences were aligned and compared using BioEdit software (version 7.2.3) [33]. Nucleotide sequence data were analysed using MEGA7 software [34]. The evolutionary history was inferred using the neighbor-joining model with the p-distance method, and the dataset was subjected to bootstrap analysis of 1000 replicates. MLST alleles were assigned to the RIRDC MLST database (https://pubmlst.org/pmultocida/). For making a comparison on a wider range, a phylogenetic tree was constructed using all STs available in the RIRDC MLST database. Then we cut the tree showing only branches containing STs of our isolates together with previously identified STs.

2.3. Antimicrobial resistance

Antimicrobial resistance was tested using minimal inhibitory concentration (MIC) test strips (Liofilchem, Roseto, Italy). Susceptibility to 14 antimicrobial agents (ampicillin, cefazolin, cefpodoxime, streptomycin, gentamycin, tetracycline, doxycycline, erythromycin, tilmicosin, clindamycin, chloramphenicol, florfenicol, sulfamethoxazole and enrofloxacin) was tested. *Escherichia coli* ATCC 25922 served as a

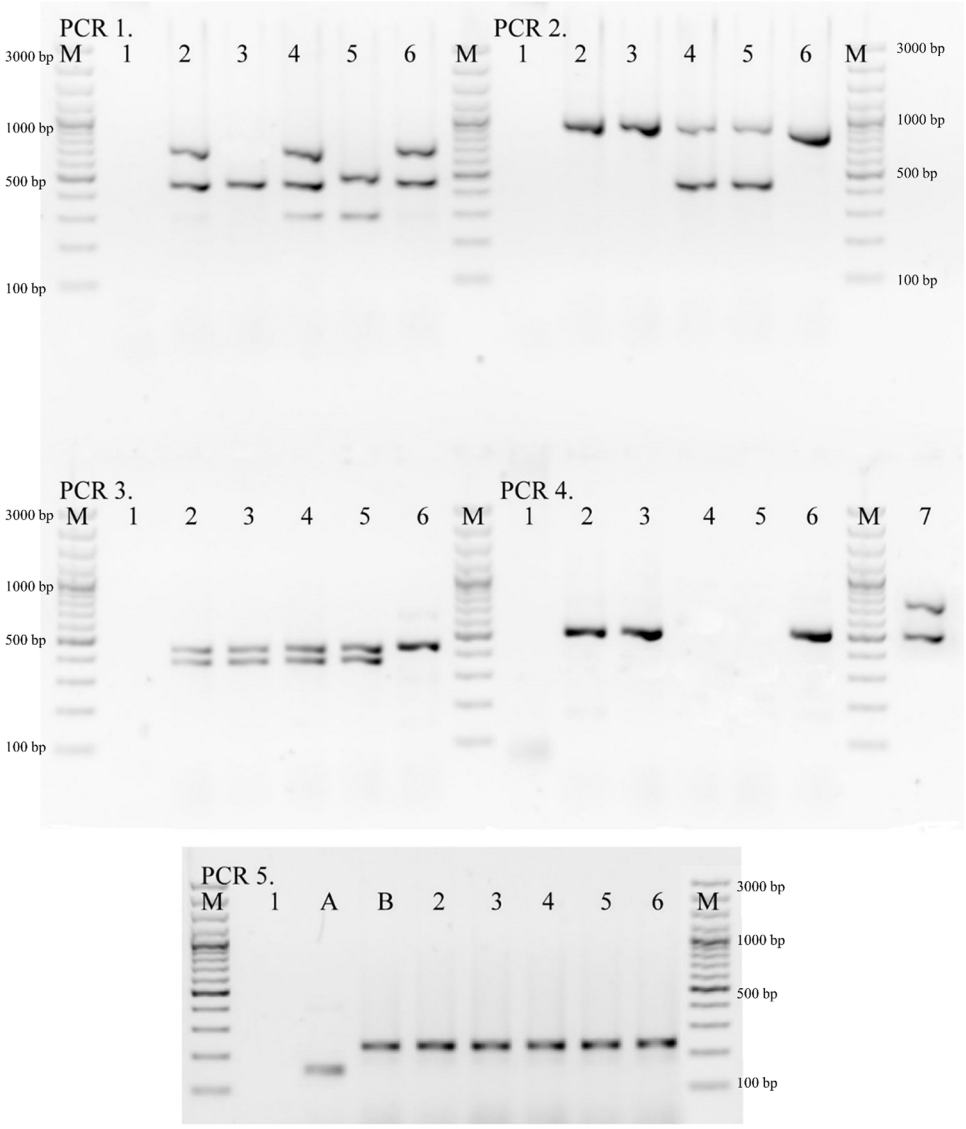

Fig. 1. Detection of virulence associated genes of *P. multocida* by PCR in agarose gel. Electrophoresis was performed in 1.5% agarose gel (TopVision Agarose, Lonza Group Ltd., Basel, Switzerland) and a 100–3000 bp DNA ladder (GeneRuler 1 kb Plus, Thermo Scientific Inc.) was used as molecular weight marker (M).
PCR 1. Detection of *hsf1* (654 bp), *hsf2* (433 bp) and *pfhA* (286 bp) in a multiplex reaction.
PCR 2. Detection of *fimA* (866 bp) and *tadD* (416 bp).
PCR 3. Detection of *hgbA* (402 bp) and *nanH* (340 bp).
PCR 4. Detection of *hgbB* (499 bp) and *tbpA* (728 bp).
PCR 5. *ptfA* allele specific PCR.
Lane 1: negative control, Lane 2: Pm242, Lane 3: Pm243, Lane 4: Pm244, Lane 5: Pm247, Lane 6: Pm249, Lane 7: positive control for both *hgbB* and *tbpA*, Lane A: positive control for *ptfA* allelic variant A (126 bp), Lane B: positive control for *ptfA* allelic variant B (217 bp)

quality control strain. The strains were cultured on Mueller–Hinton agar plates supplemented with 5% sheep blood at 37 °C for 24 h. Bacterial suspensions in phosphate-buffered saline, adjusted to a density of 0.5 McFarland, were spread onto Mueller–Hinton agar plates supplemented with 5% sheep blood using a sterile swab. An MIC test strip was placed on each plate after approximately 10 min and the plates were incubated at 37 °C for 24 h, following which the MIC values were read according to the manufacturer's instructions. We interpreted the breakpoints according to Clinical and Laboratory Standards Institute recommendations [35,36].

3. Results

The species-specific PCR confirmed the identity of all isolates of *P. multocida* used in this study (Table 1). The *toxA* gene was not detected in any of the isolates. Type A was the most prevalent capsular type detected among the human clinical isolates (14/15 strains), while the capsule biosynthesis gene *fcbD* (serogroup F) was found in only one case. All isolates from cats were of capsular type A. None of the isolates harboured *bcbD* (serogroup B), *dcbF* (serogroup D) or *ecbJ* (serogroup E) genes.

Among *P. multocida* human clinical isolates, Heddleston serovar 1 (53%) and 3 (40%) were the most frequently found serovars, while in one case Heddleston serovar 6 was detected. Among the isolates from cats, the Heddleston serovars detected were 1, 3 and 8. The result of the gel diffusion precipitin test was fully confirmed by the LPS multiplex PCR.

The most prevalent subspecies among the strains isolated from human infections was *P. multocida* subsp. *septica* (12/15 strains, 80%) and the phylogenetic lineage specific for subsp. *multocida/gallicida* was identified in three strains (20%). In *P. multocida* isolates from cats, subsp. *septica* was detected in three cases and lineage-specific nucleotide substitutions for subsp. *multocida/gallicida* were identified in two strains.

Carbohydrate fermentation patterns and ornithine decarboxylase activity recognized six different biovars among the human clinical isolates. Biovars 2, 3 and 7 were the most prevalent biovars; however, biovars 6, 9 and 10 were also detected in individual cases. Isolates from cats were assigned to biovars 2, 3, 7 or 12.

All *P. multocida* strains harboured type I and type IV fimbrial subunits (*fimA*, *ptfA* allelic variant B), the autotransporter adhesin (*hsf-2*) and, in the majority of the strains, *hgbA* (95%) and *hgbB* (90%) were also detected (Fig. 1), coding for haemoglobin binding proteins and the neuraminidase coding gene *nanH* (95%). The transferrin binding protein encoded by the *tbpA* gene could not be detected in the strains tested. In addition, some adhesion-related genes were identified in low

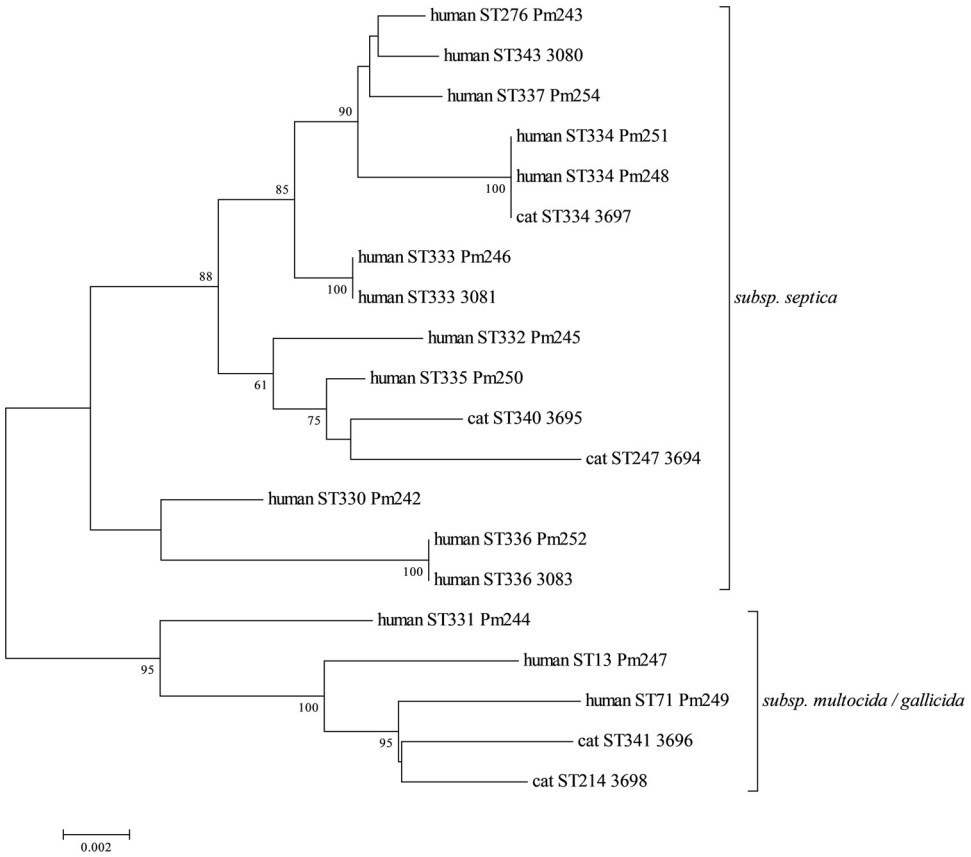

Fig. 2. Evolutionary relationships between *P. multocida* isolates obtained from cats and humans. The evolutionary history was inferred using the neighbour-joining method. Evolutionary analyses were conducted using MEGA7 software [35].

prevalence, such as those encoding the autotransporter adhesin (*hsf-1,* 20%), filamentous haemagglutinin (*pfhA,* 10%) and tight adherence protein D (*tadD,* 5%).

MLST analysis classified the 20 *P. multocida* strains into 16 different sequence types (STs). Interestingly, in most cases (11 of the 16 STs) the allelic profile could not be matched with the known STs defined in the MLST database. We therefore assigned 11 new STs.

Only three STs were represented by more than one strain: ST333 (n = 2), ST334 (n = 3) and ST336 (n = 2). The *P. multocida* subsp. *septica* strains were shown to be closely related phylogenetically (Fig. 2). Comparison with the isolates in the RIRDC MLST database indicated that *P. multocida* subsp. *septica* strains formed a distinct phylogenetic group with high bootstrap support values within the species *P. multocida* regardless of the host species. A neighbour-joining dendrogram was constructed to display the genetic distance of these STs (Fig. 3).

Based on MIC values (Table 2), the strains showed resistance to erythromycin, clindamycin and sulfamethoxazole, and with two exceptions, resistance to tilmicosin was also detected. Each strain was susceptible to ampicillin and other commonly used antimicrobials including streptomycin, gentamycin, tetracycline, doxycycline, cefazolin, cefpodoxime, chloramphenicol, florfenicol and enrofloxacin.

4. Discussion

P. multocida strains isolated from humans and cats were serotyped and characterized according to their subspecies, capsular polysaccharide and virulence-associated genes. Their clonal relatedness was also investigated by MLST.

In accordance with the data in the literature, type A proved to be the most prevalent capsular serogroup among *P. multocida* strains from cats and humans, and to the best of our knowledge, this is the first detection of capsular type F in a human case [5,24]. The dominant serovars among human and feline *P. multocida* isolates were A:1 and A:3, which widely occur in all of this bacteria's host species [5]. Interestingly, in two cases we identified the rarely reported Heddleston serovars 6 and 8, respectively.

Subspecies differentiation is considered an important epidemiological marker of *P. multocida*. In nearly all hosts, subsp. *multocida* is the most frequently detected subspecies [17,18,37]. As an exception, strains from cats and dogs usually belong to subsp. *septica* [38]. In this study, subsp. *septica* was also identified as the predominant subspecies in the human clinical isolates.

In an earlier study, Holst et al. [13] identified *P. multocida* subsp. *multocida* as the most commonly recovered subspecies from infected humans. However, they used traditional biochemical tests for the differentiation of *P. multocida* subspecies, which have some inconsistencies due to the range of sorbitol and dulcitol fermentation properties amongst these strains [39]. In our work, we determined the main phylogenetic lineages of *P. multocida* by PCR-RFLP based on 16S rDNA polymorphisms. Biochemical profile-based subspecies differentiation is not always accurate [30,38,40], and we also found sorbitol positive subsp. *septica* strains among our isolates (biovars 2 and 3). If only fermentation properties had been taken into account, these strains would have been misidentified as *P. multocida* subsp. *multocida* (Table 1.). In a previous study, Chen et al. [41] noticed that the majority of the strains recovered from respiratory infections in humans were *P. multocida* subsp. *multocida*, while *P. multocida* subsp. *septica* strains dominated isolates from wound infections. Donnio et al. [42] examining a larger number of strains of *P. multocida* recovered from human patients found that *P. multocida* subsp. *multocida* was the most common subspecies (87.5%) equally occurring in both wound infections and respiratory

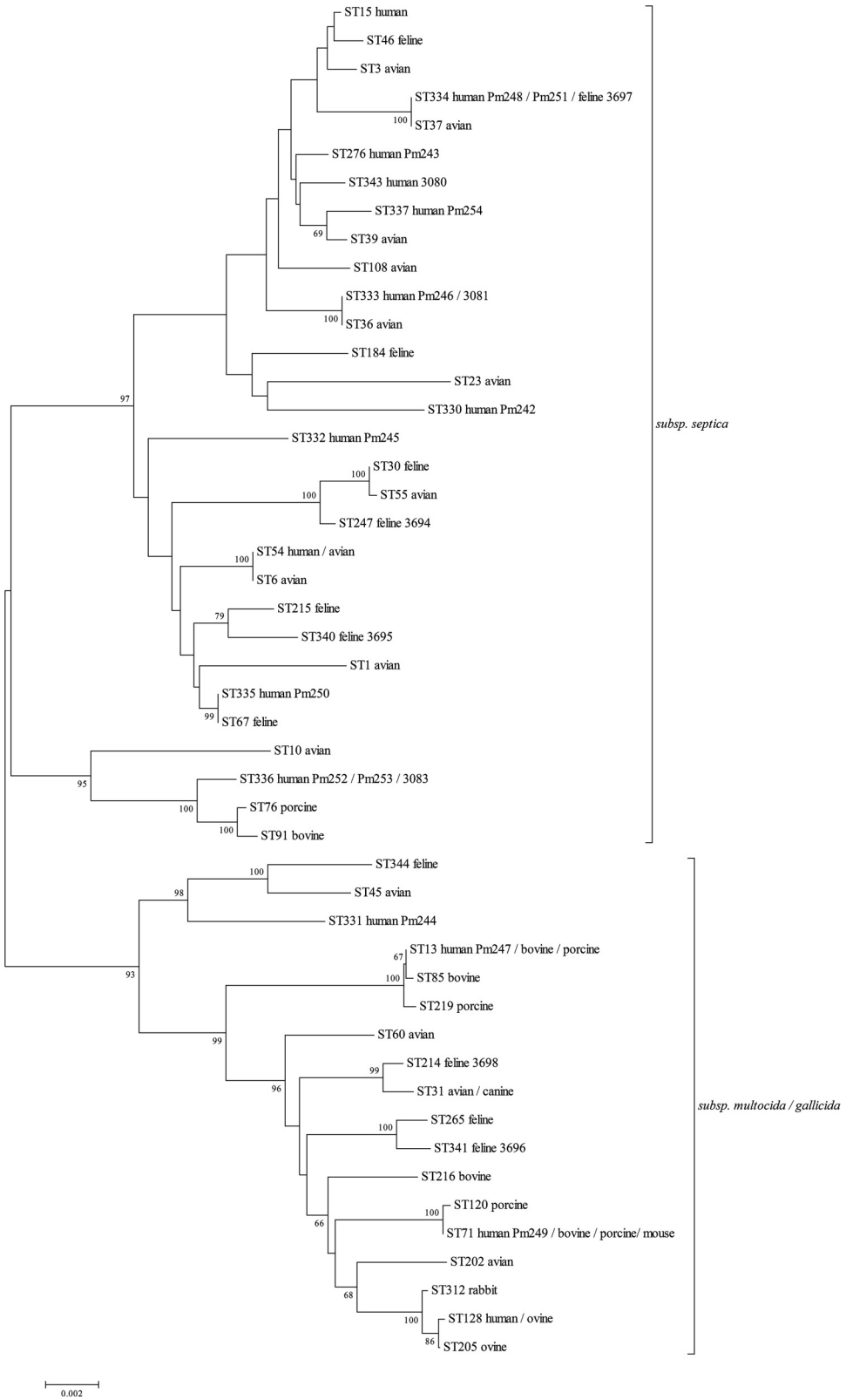

Fig. 3. Evolutionary relationships among *P. multocida* strains from various hosts and disease phenotypes. The evolutionary history was inferred using the neighbour-joining method. Evolutionary analyses were conducted using MEGA7 software [35].

tract infections. In contrast, nearly all *P. multocida* subsp. *septica* strains were isolated from bite wounds and rarely in respiratory or systemic infections [42]. The majority of our isolates represented *P. multocida* subsp. *septica*, and were recovered from infected wounds, while the only isolate from the respiratory tract belonged to *P. multocida* subsp. *multocida*. These findings provide further evidence that *P. multocida* subsp. *septica* is more commonly found in soft tissues than to other sites of the body.

With only two exceptions (Pm242 and Pm252), all *P. multocida* subsp. *septica* isolates exhibited the same virulence gene profile. These

Table 2
Susceptibility rates of *P. multocida* isolates form human and cat origin. All values are expressed as μg/ml.

Antibiotics	Human isolates			Cat isolates		
	MIC50	MIC90	Susc. %[a]	MIC50	MIC90	Susc. %[a]
Ampicillin	0.5	0.5	100	0.38	0.5	100
Cefazolin	0.75	0.5	100	0.75	1	100
Cefpodoxime	0.064	0.5	100	0.064	0.5	100
Streptomycin	32	32	100	32	32	100
Gentamycin	4	4	100	4	4	100
Tetracycline	0.19	0.25	100	0.125	0.19	100
Doxycycline	0.25	0.38	100	0.19	0.38	100
Erythromycin	12	32	0	12	16	0
Tilmicosin	32	32	13	32	32	0
Clindamycin	256	256	0	48	128	0
Chloramphenicol	1.5	4	100	3	3	100
Florfenicol	0.75	1	100	0.75	1	100
Sulfamethoxazole	256	256	0	256	256	0
Enrofloxacin	0.047	0.094	100	0.064	0.125	100

[a] Percentages of susceptible strains.

strains harboured genes encoding *ptfA* (allelic variant B), *fimA*, *hsf-2*, *nanH*, *hgbA* and *hgbB*, while genes encoding *tbpA*, *pfhA*, *hsf-1* and *tadD* were not detected. By contrast, strains belonging to the subsp. *multocida/gallicida* lineage showed greater diversity among virulence genes. Based on the seven genes in common across the two studies, Ewers et al. [24] described a similar virulence gene pattern to that we found to be characteristic of *P. multocida* subsp. *septica* strains; however, they did not determine the subspecies and included only two strains of human origin in their study. Recent research demonstrated the existence of two independent iron acquisition strategies in *P. multocida*, with the haemoglobin and transferrin binding protein coding genes harboured by *P. multocida* often being associated with certain host species [24]. Transferrin binding proteins appear to be specific to isolates from ruminants [43]. Haemoglobin binding proteins A and B facilitate the increased proliferation of the bacterium by acquiring hemin from the host, and they are prevalent among *P. multocida* isolates from different host species. Our data confirmed the findings by Ewers et al. [24] that the *hgbA* and *hgbB* occur widely among isolates from humans and cats.

Our MLST results confirmed the previous finding [44] that *P. multocida* subsp. *septica* and subsp. *multocida* strains were forming two distinct clusters, with a number of newly described STs, and considerable diversity within each cluster. On the other hand, although there are many human and feline isolates in both clusters, there are a number of avian isolates and single porcine and bovine isolates as well. Moreover, for a number of the strains (e.g. 3080, 3083, Pm242, Pm 243, Pm246, Pm252, Pm254) the closest relative strains as determined by MLST are bovine/porcine or avian not feline. Based on the results of the present study, *P. multocida* isolates from humans and cats have shown little evidence of shared genotypes. The review of the RIRDC MLST database of *P. multocida* strains from different hosts suggests that similar strains are circulating across host species [45,46].

The antimicrobial susceptibility of *P. multocida* strains from humans has been evaluated in several studies, and the resistance patterns remain largely unchanged over time. Penicillin, later generation cephalosporins, tetracyclines, chloramphenicol and fluoroquinolones seem to be generally effective against *P. multocida* isolates from humans, whereas such strains are typically resistant to clindamycin and sulphonamides [7,9,47,48]. Recently, resistance to macrolides has emerged in *P. multocida* [49]. Our results are in accordance with these previous observations, and the susceptibility profiles of *P. multocida* strains from humans and cats were found to be highly similar. Traditionally, penicillin or ampicillin were the antibiotics primarily recommended to treat *P. multocida* infection in humans [7,9], although the emergence of β-lactamase-producing strains has also been described [50]. All strains in this study, including the cat-derived isolates, have been proven to be susceptible to ampicillin. To date, no resistance to tetracycline and doxycycline has been reported in clinical isolates of *P. multocida* from humans or pet animals, and all of our strains were susceptible to both antibiotics. By contrast, resistance to tetracyclines has been described in strains from pigs and ruminants [51,52]. Furthermore, high rates of resistance to streptomycin, sulphonamide and chloramphenicol have been reported in *P. multocida* isolates of porcine and bovine origin [53–55]. These findings warn us that resistance may emerge in isolates from other host species including human beings as well. Similar to previous findings [54,55], strains susceptible to sulphonamides were not identified in the present study.

In summary, our results show that ampicillin, aminoglycosides, tetracyclines, later generation cephalosporins, phenicols and enrofloxacin may be still effective against *P. multocida* infections in humans. However, the application of macrolides, lincosamides and sulfamethoxazole should be avoided in the treatment of such infections.

In conclusion, some of the common characteristics (like virulence profile and antibiotic sensitivity pattern) shared by *P. multocida* strains isolated from humans and cats support the view that domestic cats serve as a major reservoir for *P. multocida*. This is partially contradicted by the MLST results, however, the opportunity for regular contacts with domestic animals further strengthen this presumption. The relatively frequent isolation of *P. multocida* from human cases underlines the zoonotic importance of this pathogen, and encourages its further examination from this aspect with special regard to the possible sources of infection.

Conflict of Interest Statement

We declare that the authors have no conflict of interests.

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