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Research Paper

Antimicrobial resistance and virulence gene profiles in *P. multocida* strains isolated from cats

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Abstract

Cats are often described as carriers of *Pasteurella multocida* in their oral microbiota. This agent is thought to cause pneumonia, conjunctivitis, rhinitis, gingivostomatitis, abscess and osteonecrosis in cats. Human infection with *P. multocida* has been described in several cases affecting cat owners or after cat bites. In Brazil, the cat population is approximately 21 million animals and is increasing, but there are no studies of the presence of *P. multocida* in the feline population or of human cases of infection associated with cats. In this study, one hundred and ninety-one healthy cats from owners and shelters in São Paulo State, Brazil, were evaluated for the presence of *P. multocida* in their oral cavities. Twenty animals were positive for *P. multocida*, and forty-one strains were selected and characterized by means of biochemical tests and PCR. The *P. multocida* strains were tested for capsular type, virulence genes and resistance profile. A total of 75.6% (31/41) of isolates belonged to capsular type A, and 24.4% (10/41) of the isolates were untypeable. None of the strains harboured *toxA, tbpA* or *pfhA* genes. The frequencies of the other genes tested were variable, and the data generated were used to build a dendrogram showing the relatedness of strains, which were clustered according to origin. The most common resistance profile observed was against sulfizoxazole and trimethoprim-sulphamethoxazole.

Key words: cat, *Pasteurela multocida*, virulence genes, resistance, isolation.

Introduction

Pasteurella multocida is an important pathogen that infects a wide range of animal hosts and is a member of the microbiota of the superior respiratory tract of different animal species (Dziva *et al.*, 2008). Cats are frequently described as healthy carriers of *P. multocida*. Because of their hunting habits and because they are usually involved in fights causing scratches or bites, the carriers spread the bacterium among the cat population.

Cat bites often infect humans with *P. multocida,* resulting in cellulitis and lymphangitis, sometimes complicated by abscess formation, peritonitis and septic arthritis. Case reports of infection by *P. multocida* in patients with a compromised immune response in contact with cats without bite history are becoming common (Hey *et al.*, 2012; Sol *et al.*, 2013).

P. multocida is also described as a causative agent of pneumonia, conjunctivitis, rhinitis, gingivostomatitis, abscess and osteonecrosis in cats (Ewers *et al.*, 2006). Dolieslager *et al.* (2010) report that *P. multocida* is significantly more prevalent in cats presenting gingivostomatitis than in normal cats and can be of etiological significance in this disease.

Capsular serotypes A, D and F are the most frequently isolated serotypes from cats and different virulence factors were described by Ewers *et al.* (2006) in cat strains. The pathogenicity of *P. multocida* has been associated with different virulence factors, such as capsules, adhesins, toxins, siderophores, sialidases and outer membrane proteins.

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These virulence factors improve the colonization and invasion of the host, the avoidance or disruption of host defense mechanisms, injury to host tissues, and/or stimulation of a noxious host inflammatory response (Tang *et al.*, 2009).

This is the first report of *P. multocida* isolation from carrier cats in Brazil. The aim of this study was evaluate the occurrence of *P. multocida* in healthy cats, determine the resistance profile and investigate the presence of the virulence genes encoding for outer membrane and porin proteins (*oma87, ompH, plpB, psl*), adhesins (*ptfA, fimA, hsf-1, hsf-2, pfhA, tadD*), neuraminidases (*nanB, nanH*), iron acquisition related factors (*exBD, tonB, fur, pmHAS, tbpA, hgbA, hgbB*), superoxide dismutases (*sodA*, *sodC*), dermonecrotoxin (*tox*A), and hyaluronidase (*pmHAS*).

Material and Methods

Sample collection and processing

The samples were collected in February 2008 and February 2011 with sterile swabs from the gingiva of one hundred and ninety-one cats from owners and shelters in São Paulo State. The swabs were placed in Amies transport medium and kept under refrigeration for 24 h until analysis (Copan Diagnostics Inc., CA, USA).

Each swab was plated on tryptic soy yeast extract agar (Difco-BBL) supplemented by 5% of defibrinated sheep blood and incubated at 37 °C for 24 h. From each plate, one to five colonies with morphology suggestive of *P. multocida* were selected. Colonies were identified using standard biochemical procedures, including the production of catalase, oxidase, and indol, urease activity, the production of ornithine decarboxylase, carbohydrate fermentation (Mutters *et al.*, 1989) and PCR for the detection of the species-specific gene fragment *kmt* (Townsend *et al.*, 1998).

Antimicrobial susceptibility testing

The susceptibility profile was established by a disc diffusion test as recommended by the Clinical and Laboratory Standards Institute (VET01-A4, 2013). The antimicrobial agents tested included ceftiofur, penicillin, amoxicillin, flofenicol, norfloxacin, enrofloxacin, ciprofloxacin, tetracycline, doxycycline, sulfizoxazole, trimethoprim-sulphamethoxazole and erythromycin (Oxoid Ltd., Cambridge, UK). The reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms in all antimicrobial susceptibility tests. There are no CLSI approved breakpoints applicable specifically to feline *Pasteurella multocida;* therefore, most of the values used here originated from values described in CLSI document VET01-A4 and supplement VET01-S2. The breakpoints used for doxycycline, ciprofloxacin and norfloxacin were adopted from CLSI document M100- S19 (2009).

DNA preparation

Bacteria were cultured overnight in brain hearth infusion (BHI) broth at 37 °C and 200 μL of this bacterial suspension was submitted to the DNA extraction procedure described by Boom *et al.* (1990).

PCR analyses and gel electrophoresis

P. multocida strains were evaluated by PCR for the identification (using the *kmt* gene) and presence of the capsule biosynthesis genes *capA, B, D, E* and *F* as described by Townsend *et al.* (1998). The virulence-related genes *oma87, ompH, plpB, psl, ptfA, fimA, hsf-1, hsf-2, pfhA, tadD*, *nan*B, *nan*H, *exBD/tonB, fur, pmHAS, tbpA, hgbA, hgbB*, *sodA*, *sodC*, *tox*A, and *pmHAS* were described by Ewers *et al.* (2006) and Tang *et al.* (2009). The combination of different genes in multiplex reactions was standardized in this study (Table 1). The following *P. multocida* strains were used as positive controls: ATCC 12945, ATCC 12948 and NCTC 10323.

For all reactions, 5 μL of DNA template was added to the 45 μL mixture containing 20 pmoles of each primer pair, 1.5 mM of $MgCl_2$, 200 mM of each dNTP, 1 U of Taq DNA polymerase (Fermentas Inc., Maryland, USA), 1X PCR buffer and ultra-pure water. The PCR conditions were carried out according to the respective authors' protocols. The amplified products were subjected to electrophoresis in a 1.5% agarose gel, stained with BlueGreen® (LGC Biotecnologia, Cotia, SP, Brazil), and identified using a 100 bp DNA ladder.

Statistical analysis

Relatedness among *P. multocida* strains was determined by a comprehensive pair-wise comparison of different gene combinations using the Dice coefficient by means of the Bionumerics 6.6 software (Applied Maths NV, Sint-Martens-Latem, Belgium) to generate the dendrogram.

Table 1 - Distribution of genes evaluated in single gene PCR or multiplex PCR and the size of the expected products.

PCR	Gene	Amplicon (bp)
E1	kmt 1	560
C1	hyaD-hyaC, bcbD, dcbF, ecbJ, fcbD	1.044, 760, 657, 511, 851
FV1	hgbA, ptfA, hgbB, exbBD/ tonB	419, 488, 788, 1144
FV2	nanH, psl, nanB	360, 439, 584
FV3	ompH, oma87	438, 949
F4	pfhA, sodC	275, 235
FV5	sodA	361
FV 6	tbPA	728
FV 7	fimA, pmHAS, OmpA	866, 430, 201
FV 8	hsf1, hsf2, fur	654, 433, 244
FV 9	tadD, plpB	416, 282

Results

Of the animals, 10.5% were positive for *P. multocida* isolation (20/191) and forty-one strains were selected for PCR characterization and antimicrobial susceptibility testing. Thirty-one strains were characterized as type capsular A and ten were untypeable using PCR as described by Towsend *et al.* (1998). The frequency of different virulence genes is presented in Table 2 and the profiles generated through gene combinations are shown in Figure 1. In the dendrogram, the numbers that identify the strains are representative of the examined animals. The strains from the same animals in several cases presented different virulence gene profiles. Strains 94.1, 94.3 and 94.4, for example, were isolated from the same cat, but present a different combination of virulence genes and different resistance patterns.

The frequency of antimicrobial resistance is described in table 3. Of the forty-one selected strains, 12.1% (5/41) were susceptible to all tested drugs, and 87.8% (36/41) were resistant to at least one drug tested. The resistance was more frequent to trimethoprim-sulphamethoxazole (75.6%), followed by sulfizoxazole (60.9%).

Discussion

Although *P. multocida* is frequently reported in feline oral microbiota and isolated in cat bites (Goldstein *et al.*, 2012), the literature contains no data about the occurrence, presence of virulence genes or resistance profiles in *P. multocida* strains from cats in Brazil.

The frequency of *P. multocida* observed in this study (10.4%) was much lower than that reported by Freshwater (2008), who found 89.9% of positive samples in the gingival mucosa of cats (368/409). This difference could be related to the culture media used, interference of competitive oral microbiota or geographical differences.

In this study, tryptic soy yeast extract agar containing 5% of defibrinated sheep blood without antibiotics was used, while Freshwater (2008) used the same media with 5 mg/L of clindamycin. The choice of do not include antibiotic at the culture media in this study, was done because in a pilot study a poor growth of bacteria on blood agar plates was observed, instead an overgrowth of bacteria from microbiota that could make the isolation of *P. multocida* colonies more difficult (data not shown).

P. multocida infections are often treated with broad-spectrum antimicrobials (Kehrenberg *et al.*, 2001). Resistance profile showed that cephalosporin, fluorquinolones and florfenicol are the most efficient drugs to be used

Table 2 - Frequency of protein-coding genes and virulence factors in *P. multocida* strains isolated from cats.

Gene	Virulence factor	No of positives (%)
toxA	Dermonecrotic Toxin	0/41 (0.0)
pfhA	Filamentous hemagglutinin	0/41 (0.0)
hgbA	Hemoglobin-binding protein	25/41 (60.9)
hgbB	Hemoglobin-binding protein	35/41 (85.3)
exbBD-tonB	Iron acquisition	39/41 (95.1)
nanH	Neuraminidase	34/41 (82.9)
Psl	Porin	38/41 (92.6)
nanB	Neuraminidase	40/41 (97.5)
oomph	Outer membrane protein H	39/41 (95.1)
oma87	Outer membrane protein 87	41/41 (100.0)
ptfA	Type 4 fimbriae	26/41 (63.4)
soda	Superoxide dismutase	35/41 (85.3)
sodC	Superoxide dismutase	21/41 (51.2)
tbpA	Transferrin binding protein	0/41 (0.0)
fimA	Fimbriae	25/41 (60.5)
hsf1	Autotransporter Adhesin	5/41/ (12.1)
hsf2	Autotransporter Adhesin	11/41 (26.8)
tadD	Putative nonspecific tight adherence protein D	9/41 (21.9)
Fur	Ferric uptake regulation protein	7/41 (17.0)
pmHAS	Hyaluronan synthase	26/41 (63.4)
OmpA	Outer membrane protein A	14/41 (34.1)
plpB	Lipoprotein B	20/41 (48.7)

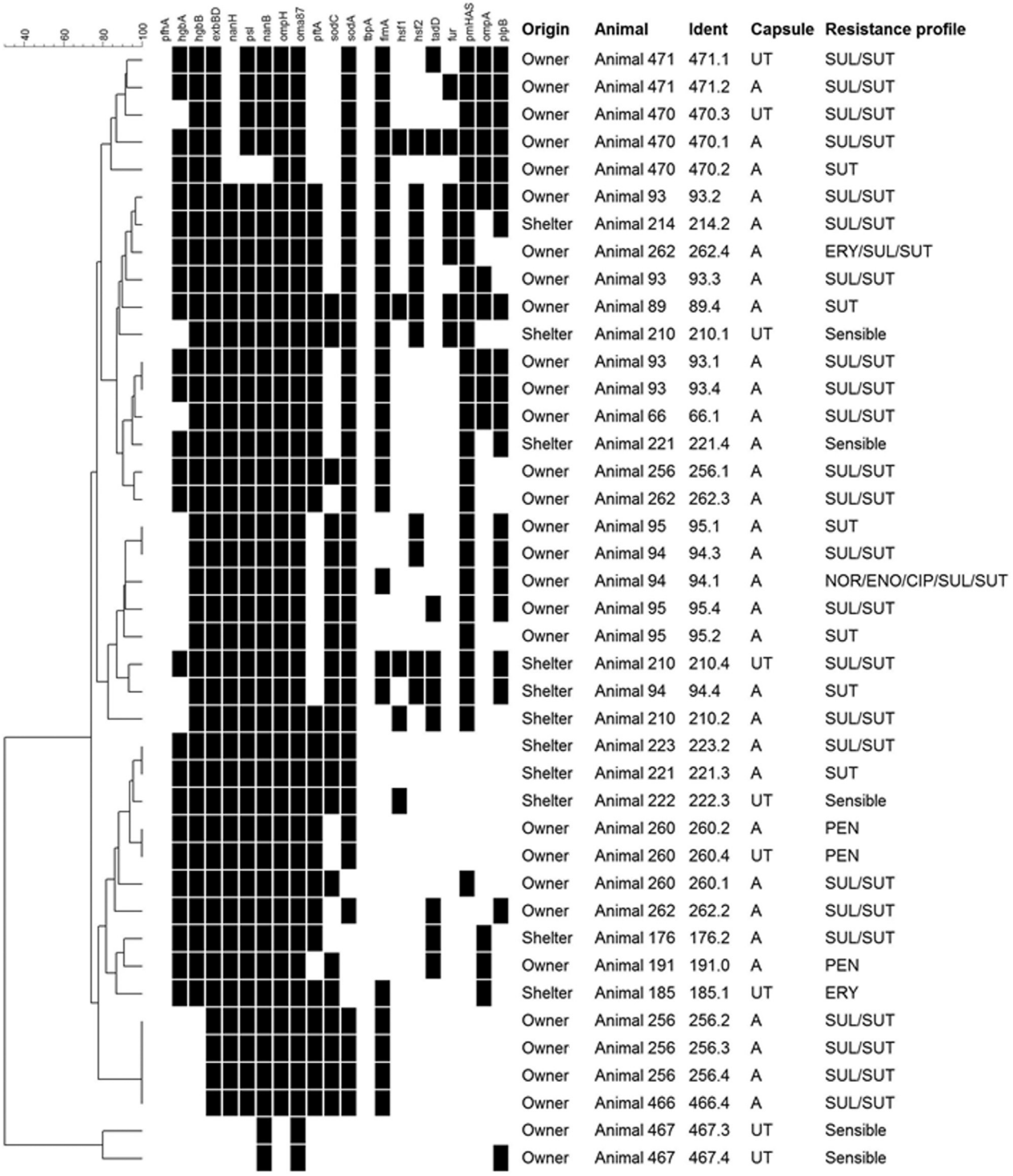

Figure 1 - Dendrogram representing the relatedness of *P. multocida* strains isolated from cats according to their virulence profile.

against *P. multocida*. Similar results have been previously described in Brazil, France, North America and Japan (Rigobelo *et al.*, 2013; Kehrenberg *et al.*, 2001; Salmon *et al.*, 1995; Yoshimura *et al.*, 2001). Resistance to sulfizoxazole and trimethoprim-sulphamethoxazole has been described previously (Tang *et al.*, 2009).

One strain showed an unusual resistance pattern, being resistant to norfloxacin, enrofloxacin, ciprofloxacin, sulfizoxazole and trimethoprim-sulphamethoxazole. Two strains were resistant to erythromycin. Resistance to fluorquinolones is rare in *P. multocida* strains and is most likely due to mutations in the genes *gyrA* and *parC,* encoding

Table 3 - Frequency of antimicrobial resistance in *P. multocida* strains isolated from cats, disk content and breakpoints used in this study.

Antimicrobial	Disk content	Zone Diameter Breakpoints*(mm)			Nº of resistant strains (%)
	(μg)	S	I	R	
Ampicillin	10	≥ 17	14-16	≤ 28	0/41 (0.0)
Ceftiofur	30	≥ 21	18-20	≤ 17	0/41 (0.0)
Cotrimoxazole	1.25/23.75	≥ 16	11-15	≤ 10	31/41 (75.6)
Doxycycline**	30	≥ 16	13-15	≤ 12	0/41 (0.0)
Enrofloxacin	5	≥ 23	17-22	≤ 16	1/41 (2.4)
Norfloxacin**	10	≥ 17	13-16	≤ 12	1/41 (2.4)
Ciprofloxacin**	5	≥ 21	16-20	≤ 15	1/41 (2.4)
Erythromycin	15	≥ 23	14-22	≤ 13	2/41 (4.9)
Florfenicol	30	≥ 22	19-21	≤ 18	0/41 (0.0)
Penicillin	10	≥ 29	-	≤ 28	3/41 (7.3)
Sulfizoxazole	300	≥ 17	13-16	≤ 12	25/41 (60.9)
Tetracycline	30	≥ 19	15-18	≤ 14	0/41 (0.0)

* Trimethoprim/sulphamethoxazole.
** Breakpoints from CLSI - M100- S19 (2009).

DNA gyrase and topoisomerase IV (Michael *et al.*, 2012). Hendriksen *et al.* (2008) evaluated antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries and described a resistance rate to fluorquinolones of 4.2% in France in 2002 and a rate of 6.3% in the Netherlands in 2004. Portis *et al.* (2012) reported that *P. multocida* isolated from cattle in the United States and Canada had rates of resistance to danofloxacin varying from 8.5 to 13.1 in 2004 and 2009. In the same study, enrofloxacin resistance rates were 2.1 and 3.5 in 2008 and 2009, respectively. The use of fluorquinolones in veterinary clinics, hospitals and shelters is high in Brazil, but it could not be directly related to this resistance profile because it was observed in only one animal, and it was not possible to evaluate its health history.

Erythromycin is a member of the macrolide class of antibiotics. Mechanisms of resistance to this class of antibiotics in *P. multocida* have been well studied. There are no descriptions of resistance to this antibiotic in companion animals (Schwarz *et al.*, 2007), but Tang *et al.* (2009) describe 6% of *P. multocida* strains resistant to erythromycin among 233 strains from swine in China.

Using PCR, 75.6% (31/41) of the strains were positive for capsular type A, in agreement with reports in the literature as the most common capsular type in cats (Arumugam *et al.*, 2011). A similar frequency of untypeable strains (24.4%) was also found using the reaction described by Towsend *et al.* (1998) compared to the description of Arumugam *et al.* (2011). These authors reported that 19.3% (22/114) of *P. multocida* strains were untypeable using PCR, while other studies describe frequencies varying from 2 to 9% (Davies *et al.*, 2003a; 2003b; Ewers *et al.*, 2006; Jamaludin *et al.*, 2005; Tang *et al.*, 2009).

Using traditional serotyping, Arumugam *et al.* (2011) described a significantly higher percentage of untypeable strains than observed by PCR, reaching 48.2% (55/114). The expression of capsular type is a critical point for the virulence potential of *P. multocida* strains and can be influenced by different factors, such as antimicrobial presence, iron loss and multiple in vitro passages (Steen *et al.*, 2010). However, explanations for the absence of capsule-related loci (A, B, D, E or F) were not found in the literature.

The frequency of virulence genes varies from zero, for example for iron acquisition-related protein (*tbpA*), filamentous hemagglutinin (pfhA) and dermonecrotoxin, to 100%, for example for outer membrane protein (*oma87*). Sina *et al.* (2006) report that the *tbpA* gene is related to bovine infections and that dermonecrotoxin is involved in atrophic rhinitis in swine, justifying the absence of these genes in cat strains. In contrast to this study, Ewers *et al.* (2006) report that 18.5% of *P. multocida* strains isolated from cats are positive for filamentous hemagglutinin (*pfh*A), but a correlation of this factor with clinical pasteurellosis was established only in bovine strains.

The high frequency of genes encoding for outer membrane proteins (*oma87*- 100%, *ompH* - 95.1%) is very relevant to the invasion potential of the tested strains because OMPs act as selective barriers preventing the entry of toxic molecules in the cell, which is crucial to bacterial survival in different environments. At the same time, these proteins play different roles in bacteria, such as nutrient absorption, importation and exportation of molecules and a close interaction with host tissue (Hatfaludi *et al.*, 2010).

Other genes detected at a high frequency were iron acquisition-related factors (*exBD/ tonB* - 95.1%, *hgbA* - 60.9%, *hgbB* - 85.3%), adhesins (*ptfA* - 63.4, *fimA* - 60.5%, *hsf-1* - 12.1%, *hsf-2* - 26.8%, *pfhA* - 0%, *tadD* - 21.9%), porin proteins (*plpB* - 48.7%, *psl* - 92.6%), neuraminidases (*nanB* - 97.5%, *nanH* - 82.9%), and superoxide dismutase (*sodA* - 85.3%, *sodC* - 51.2%, *fur* - 17%, *pmHAS* -34.1%, *tbpA* - 0%).

In addition to the zoonotic aspects, it is important to remember that in cats, *P. multocida* is one of the most frequent pathogens present in infected skin wounds and subcutaneous abscesses. It is a common pyothorax-producer in cats and has been associated with spinal empyema and meningoencephalomyelitis (Lloret *et al.*, 2013).

The results described in this study suggest that cats can carry *P. multocida* in the oral cavity and that isolated strains have considerable virulence gene baggage associated with antimicrobial resistance against drugs used in human and veterinary medicine. Considering the high proximity of cats and owners, the habits of kissing the animals or allowing them free access to the bedroom or kitchen environment, it is important to emphasize the need for hygienic measures to prevent contamination by this agent. In addition to bites and licks, close contact with cats has been enough to cause infection in humans (Lloret *et al.*, 2013).

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