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Short Communication

Prevalence and Risk Factor Analysis of Haemoplasmas Infection in Cats from Lahore

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Authors' Contribution
FI had designed the study and prepared the manuscript. AKM collected the samples. MS, OI, MS and SK conducted the laboratory investigations and analyzed the data. AP performed the phylogenetic analysis. HV and CM provided the technical support and revised the manuscript.

Key words
***Mycoplasma haemofelis, Candidatus Mycoplasma haemominutum*, Cat, 16S rDNA, PCR**

ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are obligate Gram-negative bacteria that target red blood cells, and infect a wide range of hosts including cats, dogs, domestic ruminants, pigs, rodents and humans. The present study was designed for the molecular detection of *Mycoplasma haemofelis* and *Candidatus Mycoplasma haemominutum* in feline blood samples collected from various pet clinics in Pakistan, by Polymerase Chain Reaction (PCR), using 16S rDNA as the target sequence. Clinical and epidemiological data was collected in all animals included in the study. *M. haemofelis* and *C. Mycoplasma haemominutum* DNA was detected by PCR respectively in 6.8% (10/148) and in 18.2% (27/148) of cat blood samples. Of these, two animals were co-infected with both agents. Sequencing and phylogenetic analysis was performed in *M. haemofelis* infected samples. Analysis of risk factors revealed that risk of *M. haemofelis* and of *C. Mycoplasma haemominutum* infection was significantly higher during summer months than during the winter season ($P \leq 0.01$ in both agents). Cats older than one year of age were significantly more predisposed to *C. Mycoplasma haemominutum* infection than younger cats ($P \leq 0.001$), and fever manifestation was significantly associated with *M. haemofelis* infection ($P \leq 0.001$) in enrolled subjects. In conclusion, this study represents the first report of molecular detection of *M. haemofelis* and *C. Mycoplasma haemominutum* in cats from Pakistan. Therefore, this agents should be considered in cats from Pakistan presented with compatible clinical signs, mainly those presented during summer season, and in adult cats and cats presented with fever.

Hemotropic mycoplasmas (hemoplasmas) are gram-negative, epicellular bacterium that target erythrocytes (Aktas and Ozubek, 2018). Four hemoplasmas species, namely *Mycoplasma haemofelis*, *Candidatus Mycoplasma haemominutum*, *Candidatus Mycoplasma turicensis* and *Candidatus Mycoplasma haematoparvum*-like infect cats (Willi *et al*., 2005). Of these, *M. haemofelis* is the most pathogenic, and is an important cause of anemia in felines (Raimundo *et al*., 2016). Vectors of hemoplasmas include fleas, lice, mosquitoes and ticks. Fleas are probably the most common parasite of cats, and *Ctenocephalides felis* is reported to be the most common cat flea (Otranto and Dantas-Torres, 2010). Clinical signs of hemoplasmas infection are not specific and generally include anemia, mucosal pallor, lethargy, anorexia, weight loss and depression. Fever is also frequent, especially in the acute stage of the disease. Splenomegaly and lymphadenopathy may occur due to extra medullary haematopoiesis (Hammer and Wellman, 1999).

Feline hemoplasmas have a worldwide distribution. However, information about prevalence of hemotropic *Mycoplasma* spp. infection in cats from Pakistan is lacking. This study aimed to determine the prevalence and the risk factors of hemotropic mycoplasmas infection in cats from Pakistan.

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Materials and methods

Blood samples were collected from 148 apparently healthy or diseased client-owned cats, presented to the Veterinary Clinic of the University of Veterinary and Animal Sciences (UVAS) in Lahore, Pakistan. None of the enrolled cat was diagnosed with feline mycoplasmosis. Blood samples (approximately 2 ml) were collected from jugular vein into eppendorf tubes containing 0.5M EDTA solution as anticoagulant. Clinical and epidemiological data was collected in all animals included in the study. DNA extraction and PCR were performed in Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan. Sequencing and phylogenetic analysis were performed in blood samples positive for *M. haemofelis* at the Institute of Hygiene and Tropical Medicine of the NOVA, University of Lisbon. All the experimental protocols and animal handling procedures were approved by the ethical review board of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan (IPAB/Eth/2016-MH 163).

DNA extraction from the collected blood sample was carried by using an inorganic method as described by Qamar *et al*. (2017). Detection of *M. haemofelis* and *C. Mycoplasma haemominutum* DNA was assessed by PCR as described by Braga *et al*. (2012). In all amplification series, positive (containing genomic DNA of *M. haemofelis* and of *C. Mycoplasma haemominutum*) and negative (without DNA) controls, were included. PCR amplifications were carried out in a DNA thermal cycler (MultiGENE OptiMAX, Labnet Inc.).

To confirm the PCR results of *M. haemofelis* infection, three representative PCR products were randomly selected for DNA sequencing. PCR products were purified from agarose gel slices with NZYGelpure® (Nzytech, Portugal), and subsequently sequenced (LIGHTrun™ Sequencing Service, GATC-biotech, Germany) with the same primers used for DNA amplification. The nucleotide sequences were compared with those available in the GenBank database, NCBI web server, by using BLAST algorithm (MegaBLAST option). Multiple alignments of nucleotide sequences were performed using the iterative G-INS-i method as implemented in MAFFT v7 (Katoh and Toh, 2008). Resulting alignments was treated via Gblocks (Castresana, 2000), (using the least stringent options available) through Seaview v4.6.1 software (Gouy *et al*., 2010). The obtained DNA sequences were deposited at GenBank.

Phylogenetic tree was constructed using a maximum likelihood (ML) approach using the best fitting evolutionary model (GTR+I), on the basis of the AIC selection criterion, as suggested by jModelTest v2.0 (Darriba *et al*., 2012). Phylogenetic reconstructions based on the ML optimization criterion were carried out using Mega v6.0 (Taimura *et al*., 2013) and the stability of the obtained tree topology assessed by bootstrapping with 1000 re-samplings of the original sequence data. The final tree were edited for display using FigTree v1.4.3.

All the data is represented as Mean ± SEM. Significance level was set at $P < 0.05$. Results were statistically analyzed by statistical package Minitab (version 17). Previously described risk factors for *M. haemofelis* and *C. Mycoplasma haemominutum* infection, *i.e.* gender, age, breed, body temperature, mucous membranes color, presence of haemolysis and/or haemoglobinuria, hydration status, deworming and vaccination habits, history of ectoparasites infestation and sampling seasons were analysed by the Fischer's exact test.

Results and discussion

One hundred and forty eight animals were enrolled in the study (Table I). Information on gender was available for 143 cats, of which 77 (53.8%) were females and 66 (46.2%) were males. Ages (data available for 146 cats) ranged from 0.2 to 6 years old (mean 2.2 ± 1.2 years). Most cats were domestic short-haired cats (95.6%).

M. haemofelis DNA was detected in 10 out of 148 (6.8%) and of *Candidatus Mycoplasma haemominutum* detected in 27 out of 148 (18.2%) cat blood samples. Considering the 148 cats in which both agents were tested, 35 (25.0%) animals were infected with at least one hemoplasma species, and two cats (1.4%) were co-infected with both agents.

Hemotropic mycoplasmas present a worldwide distribution, and several studies have been conducted around the world regarding the prevalence of this bacterium in cats (Aragão-de-Sousa *et al*., 2013; Firmino *et al*., 2016; Kewish *et al*., 2004). Prevalence of infection presents geographical variation, probably due to differences in climatic conditions, vector prevalence and sampling season. The prevalence of *M. haemofelis* by PCR ranged from 1.49% in cats from Belem (Aragão-de-Sousa *et al*., 2013) to 35.3% in cats from Brazil (Firmino *et al*., 2016). *C. Mycoplasma haemominutum* is the most frequent feline hemoplasma in most studies, with reported prevalence ranging from 15.2% in Brazil (Alexandre- de- Santis *et al*., 2014) to 46.7% in cats from Japan (Fujihara *et al*., 2007). Similarly, in the present study, *C. Mycoplasma haemominutum* presented a higher prevalence of infection in cats from Pakistan than *M. haemofelis* (18.2% and 6.8%, respectively). Prevalence of infection detected in the present study was similar to prevalence reported in cats from Iran (Ghazisaeedi *et al*., 2014) although prevalence of infection in present study was slightly higher for *M. haemofelis* than *C. Mycoplasma haemominutum*.

Partial DNA sequence of the 16S rDNA gene of *M. haemofelis* was obtained and submitted to the EMBL/GenBank database under the Accession number KY709688. A BLAST analysis revealed nucleotide sequence identities of 99% (100% query cover; 3^{-108} E-value) with

Table I. Analysis of risk factors for *M. haemofelis* and *Candidatus Mycoplasma haemominutum* feline infection.

		Mycoplasma haemofelis			Candidatus *Mycoplasma haemominutum*		
		n	**Infected (%)**	**P-value**	**n**	**Infected (%)**	**P-value**
Gender	Male	60	06 (10%)	0.3	74	17 (23%)	0.3
	Female	75	04 (5%)		63	10 (16%)	
Age	>1 year	96	07 (7%)	1.0	44	17 (39%)	0.0001 ***
	<1 year	41	03 (7%)		96	10 (10%)	
Breed	Pure breed	8	00 (0%)	1.0	07	01 (14%)	1.0
	DSH	103	10 (10%)		114	26 (23%)	
Body temperature	Normal	120	04 (3%)	0.003 **	120	19 (16%)	0.17
	Fever	28	06 (21%)		28	08 (29%)	
Mucous membrane	Normal	136	08 (7%)	0.2	136	27 (20%)	0.1
	Pale	12	02 (17%)		12	00 (00%)	
Hydration status	Normal	146	10 (7%)	1.0	146	27 (18%)	1.0
	Dehydrated	02	00 (0%)		02	00 (0%)	
Haemolysis	Present	01	00 (0%)	1.0	01	00 (0%)	1.0
	Absent	147	10 (7%)		147	27 (19%)	
Vomiting	Present	18	01 (6%)	1.0	18	02 (11%)	0.5
	Absent	130	09 (7%)		130	25 (19%)	
Vaccinated	Yes	65	03(5%)	0.5	66	11(17%)	0.6
	No	83	07 (8%)		82	16(20%)	
Dewormed	Yes	59	03 (5%)	0.7	60	10 (17%)	0.8
	No	89	07 (8%)		88	17 (19%)	
History of ectoparasites	Present	19	02 (11%)	0.6	19	02 (11%)	0.5
Infestation	Absent	129	08 (6%)		129	25 (19%)	
Season	Winter	57	00 (0%)	0.007 **	57	04 (7%)	0.004 **
	Summer	89	10 (11%)		90	23 (25%)	

DSH, Domestic short-hair cats; P > 0.05, Non significant; P < 0.01, significant; P < 0.001, highly significant.

homologous sequences of *M. haemofelis* isolates registered in GenBank. The phylogenetic analysis of the obtained DNA sequence placed it in one stable monophyletic cluster (supported by maximum bootstrap value) along with related 16S rRNA sequences of *M. haemofelis* and *M. haemocanis* downloaded from public databases (Fig. 1).

M. haemofelis and *C. Mycoplasma haemominutum* infections were significantly more frequent in summer months than during the winter season ($P \leq 0.01$ for both agents). Our results are in agreement with those reported in cats from Brazil (Raimundo *et al*., 2016) and Italy and are probably related with the higher number of vector fleas during summer. Manifestation of fever was significantly associated with *M. haemofelis* infection ($P \leq 0.001$) during present investigation. Our results are in agreement with those of Kewish *et al*. (2004) who had reported that most cats having fever were harbouring the microorganism. Cats older than one year of age were significantly more predisposed to *C. Mycoplasma haemominutum* infection than younger cats ($P \leq 0.001$). No other significant associations were detected between presences of *M. haemofelis* or *C. Mycoplasma haemominutum* and the risk factors analysed (Table I). This result is in agreement with those of Raimundo *et al*. (2016) who had reported that adult cats had more chances to be infected by *C. Mycoplasma haemominutum* than young cats.

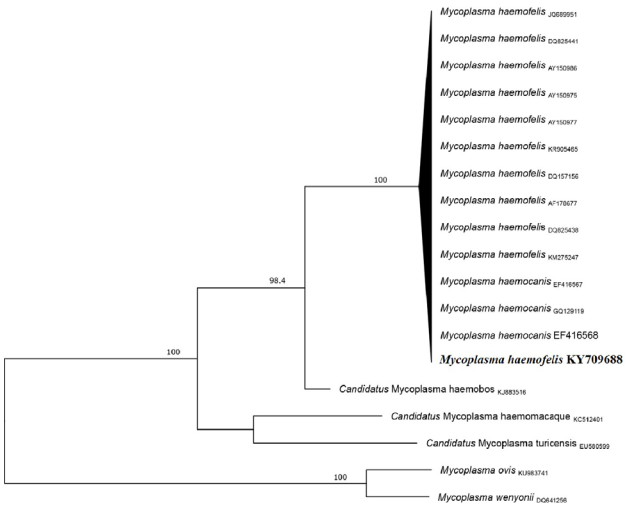

Fig. 1. Maximum likelihood phylogenetic tree (midpoint rooted) of *M. haemofelis* partial 16s rDNA gene sequence amplified from cat blood sample. The percentages of significant (≥ 75) bootstrap values of 1000 resamplings of the original data are indicated at specific branch-nodes. The size bar indicates 0.02 substitutions per site.

In the present study, cat blood samples were collected from domestic short haired cats and pure-breed cats included siamese and persian cats. Hemoplasmas infection were detected in domestic short hair, persian and siamese cats. The prevalence of *M. haemofelis* (17%) in blood of stray cats was considerably higher (P = 1) than that found in other cat breeds. Our results are in agreement with Kamrani *et al*. (2008) who had reported that the prevalence of *M. haemofelis* (47%) in blood from stray cats (n = 45) was considerably higher than in pet cats. In addition, and although not statistically significant, males were more likely to be infected with both agents, corroborating previous data obtained by Sykes *et al*. (2008) who had reported that male cats were at higher risk for hemoplasmas infection than females.

In conclusion, this is first molecular report of *M. haemofelis* and *C. Mycoplasma haemominutum* infection in cats from Pakistan. Prevalence of hemoplasmas infection detected in cats from Pakistan was considerable. Therefore, this agents should be considered in cats from Pakistan presented with compatible clinical signs, mainly those presented during summer season, and in adult cats and cats presented with fever.

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Statement of conflict of interest

Authors declare no conflict of interest.

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