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ORIGINAL ARTICLE

Prevalence and risk factor analysis of *Haemobartonella felis* in cats using direct blood smear and PCR assay

Saam Torkan · Seyed Javid Aldavood · Seeyamak Mashhady Rafie · Hossein Hejazi · Darioush Shirani · Hassan Momtaz

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Abstract The present case–control study was conducted to determine the prevalence of *Haemobartonella felis*, study the role of its risk factors and compare the different diagnostic tests. In order to achieve these goals, direct blood smear and single PCR assay were performed on blood samples from 90 cats (45 healthy controls and 45 sick cats). In addition, age, sex, breed, outdoor access, castration and density of living area were recorded in each group, and haematological analysis was performed on all blood samples. A total of 50 and 72.2 % of cats were *H. felis* positive in direct blood smear and PCR, respectively. The results showed a significant difference between control and case groups regarding the quantitative variables including fever and dehydration in direct blood smear, and tachycardia, icterus, fever and dehydration in PCR ($P<0.05$). Castration, sex, breed, access to the outside (lifestyle) and density of cat population did not significantly affect test results. The results indicate that interactive effects of various factors (risk factors of infection) can diminish the individual specific effect of each.

Keywords Cat · *Haemobartonella felis* · Smear · PCR · Iran

S. Torkan · S. M. Rafie
Department of Small Animal Internal Medicine,
Science and Research Branch, Islamic Azad University,
Tehran, Iran

S. J. Aldavood (✉)
Department of Small Animal Internal Medicine, Clinical Science,
Veterinary Faculty, University of Tehran,
Tehran, Iran
e-mail: sja@ut.ac.ir

H. Hejazi
Department of Parasitology and Mycology, School of Medicine,
Esfahan University of Medical Science,
Isfahan, Iran

D. Shirani
Department of Clinical Science, Faculty of Veterinary medicine,
University of Tehran,
Tehran, Iran

H. Momtaz
Department of Microbiology, Faculty of Veterinary Medicine,
Shahrekord Branch, Azad University,
Shahrekord, Iran

Introduction

Haemobartonella felis, the causative agent of feline infectious anaemia, has recently been reclassified as a haemotrophic mycoplasma (haemoplasma). It can cause disease as a primary pathogen or by opportunistic infection as sequelae to other diseases, particularly in those with immunosuppression (Criado-Fornelio et al. 2003). The pathogen can be identified as small coccoids, rings or strings on erythrocyte membrane or free in plasma by Giemsa staining of blood smears (Carter and Chengappa 1995). This extracellular pleomorphic bacterium attaches to the surface of feline erythrocytes (Jain and Keeton 1973; Small and Ristic 1967), causing haemolytic anaemia through extravascular destruction of erythrocytes by the mononuclear phagocyte system and intravascular lysis either by direct damage to the cell membrane or an increase in osmotic fragility or both (Maede 1979).

Two distinctly different haemotrophic mycoplasmas (*Mycoplasma haemofelis* and *Mycoplasma haemominutum*) have been identified in cats based on 16S rRNA gene sequences. The similarities of gene sequences between these organisms are only 83 %. These bacterial pathogens are sometimes present in blood from mammals such as cats, mice and dogs (Neimark et al. 2002; Penzhorn et al. 2001). These bacteria can be transmitted experimentally by parenteral or oral transfer of small amounts of infected whole blood into susceptible cats. Intrauterine transmission can also occur, and infections

can be transmitted iatrogenically via blood transfusions. However, the natural mode of transmission is believed to be via bloodsucking arthropods (such as fleas) and possibly via bite wounds.

No clinical signs may be recognizable in cats with subclinical infections and mild anaemia. The most common clinical signs in affected cats are tachypnea, depression, weakness, anorexia, weight loss, pale mucous membranes, dehydration, icterus, and splenomegaly (Foley et al. 1998; Harrus et al. 2002). Clinical signs depend on the stage of disease and the rapidity with which anaemia develops. If anaemia develops gradually, a cat may exhibit weight loss but remain bright and alert. In contrast, a precipitous drop in haematocrit (HCT) early in the disease, in association with a severe parasitaemia, causes subtle weight loss but marked mental depression and other clinical signs of anaemia (Cooper et al. 1999).

Since *H. felis* cannot be cultured, the diagnosis of infection is primarily based on cytological identification of organisms on the surface of red blood cells until recently. However, there are various factors that can cause a false-positive or false-negative result in this method (Butt 1990). Recently, feline PCR-based assays which are simpler, faster and usually more sensitive have been developed for *H. felis* detection. Specific PCR primers have been developed to differentiate between *M. haemofelis* and *M. haemominutum* infections in cats based on amplified DNA products (Jensen et al. 2001).

The first reported outbreak of *H. felis* in dogs and cats in Iran dates back to 1999 (Jafari Shoorijeh et al. 1999). There is lack of information on the relationship between *H. felis* and epidemiologic factors in Iran. Therefore, the main goal of the present case–control study was to evaluate this relationship.

Materials and methods

Sample collection and DNA extraction

The number of samples required for this study was calculated based on the previous investigations regarding the incidence of infection in domestic cats with *H. felis*, which is a minimum of 2.3 % in healthy cats and a maximum of 30 % in affected cats. Considering the reliability of 95 % and test power of 80 %, two 45-member groups of infected and control cats out of a 90-member population represented the sample group in this study. Background, age, sex, breed, outdoor access, castration and density of living place were recorded in each group. Since the most common findings among cats infected with *H. felis* include tachycardia, CRT increase, depression, weakness, anorexia, weight loss, pale mucous membranes, dehydration, icterus, fever and splenomegaly, each cat which simultaneously showed three or more of these symptoms was diagnosed to be infected and the rest were placed in the control group. The samples were further subcategorized into three age groups: <1 year, 1–3 years and >3 years.

To conduct the study, 2 ml blood was obtained from the vena cephalica antebrachii. The cats were clinically examined, and blood samples with and without anticoagulant were drawn into tubes for haematological, direct blood smear and PCR analysis. DNA was also obtained using a genomic DNA purification kit (Invitrogen, Paisley, UK) according to the manufacturer's protocol. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001).

Direct blood smear and haematology

Thin blood smears on glass slides were allowed to dry, methanol-fixed for 10 min, and stained with Giemsa. The samples were then microscopically evaluated for the visualization of the *H. felis* organisms. Also, cell count and RBC indices were evaluated in fresh anticoagulated blood by means of an electronic cell counter. The percentages of packed cell volume (PCV), mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and differential WBC counting were calculated using an electronic cell counter.

PCR assay

Primers were used that target the 16S rRNA gene, producing a 170-base pair (bp) product from *M. haemofelis* and a 193-bp amplicon from *M. haemominutum* (forward primer, 5′-ACGAAAGTCTGATGGAGCAATA-3′, and reverse primer, 5′-ACGCCCAATAAATCCGRATAAT-3′) (Jensen et al. 2001). All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran).

The PCR was performed in a total volume of 25 μL containing 2 μL of DNA sample, 0.5 mm $MgCl_2$, 0.2 mm $dNTP_{Mix}$, 0.8 μm each primer and 0.5 U/reaction of Taq DNA polymerase. Reactions were initiated at 94 °C for 2 min, followed by 45 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 30 s and a final elongation step at 72 °C for 5 min, with a final hold at 4 °C in a DNA thermal cycler (Master Cycler Gradient, Eppendorf, Germany). A negative control (sterile water) and a positive control DNA from *H. felis* were included in each amplification run. Reaction products (10 μL) were electrophoresed through 2.5 % agars' gels, stained with ethidium bromide and viewed on a UV transilluminator.

Statistical analysis

The data were analysed using SPSS software, version 15, by means of Chi-square, Fischer exact test and *t* test.

Results

Most of the infected cats showed weight loss, anorexia, depression, weakness and dehydration upon clinical examination. Gel electrophoresis of amplicons confirmed that all primer pairs specifically amplified the desired PCR products. Each PCR product was obtained as a clear band at 170 and 193 bp, generated by *M. haemofelis* and *M. haemominutum*, respectively (Fig. 1). In the total of 90 samples samplified by a single PCR for 16S rRNA gene, 65 (72.22 %) samples showed positive results. Fifty (55.56 %) and 35 (38.89 %) of these samples were positive for *M. haemofelis* and *M. haemominutum*, respectively. In addition, 20 (22.22 %) were positive for both pathogens. Our results indicate that the infection of *M. haemofelis* (55.56 %, 50 of 90) has a higher prevalence compared to *M. haemominutum* (38.89 %, 35 of 90) in the population studied. Direct microscopy on the blood smears revealed *H. felis* in 45 (50 %) cats (Fig. 2). All positive samples with the PCR assay showed a positive result on direct blood smear exam as well.

In each age group, the presence of pathogens was evaluated. Fifty-two percent of the cats below 1 year, 18 % of those between 1 and 3 years and 25 % of those above 3 years were affected. Other demographic profiles were also evaluated in this study, and their results are presented in Table 1. Except for age, none of the other demographic factors had a significant relationship with the results of the two test methods ($P<0.05$). According to the results, those cats that were below 1 year were more susceptible to infection with *H. felis* compared to the other age groups. The infected group (32.4 %) and the control group (13.5 %) have individual life cycle. Most clinical symptoms in the sick group included weakness, depression, weight loss and anorexia (Table 2). In the current study, the differences in levels of HCT and erythrocyte factors were significant. The results for different haematologic factors in both infected and control cats are presented in Table 3.

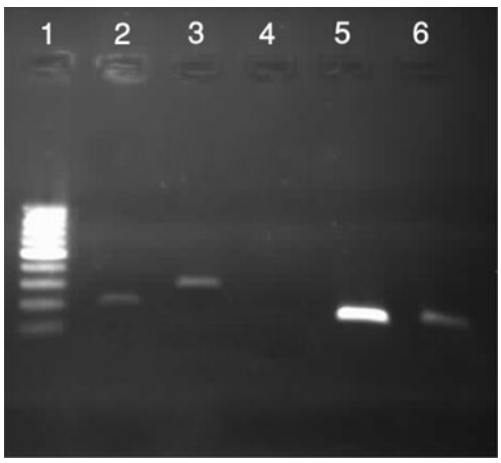

Fig. 1 Single PCR for detection of *H. felis*. Lane *1* is a 100-bp ladder; lanes *2*, *5* and *6* are positive samples for *M. haemofelis*; lane *3* is a positive sample of *M. haemominutum* and lane *4* is the negative control

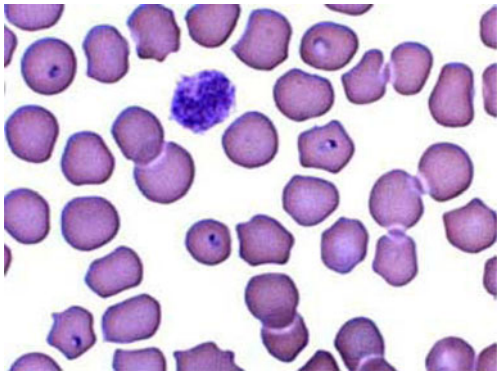

Fig. 2 Direct blood smear for detection of *H. felis*

Discussion

Untreated haemobartonellosis is a potentially lethal infection in cats. Because the symptoms are nonspecific and the diagnosis is rather difficult, the infection is commonly overlooked. Therefore, molecular techniques such as PCR have the potential to meet the needs for a better diagnostic tool for several infectious diseases caused by fastidious or slow-growing bacteria (Messick et al. 1998). Additionally, PCR results are positive 4 to 15 days after experimental infections with haemotrophic mycoplasmas, and results remain positive until appropriate antibiotic therapy is initiated (Dowers et al. 2002).

In the current study which used PCR, *M. haemofelis* and *M. haemominutum* infections were diagnosed to be 38.89 and 55.56 %, respectively. These results are comparable to results from other studies that reported a prevalence rate of 68 % for *M. haemofelis* and 22 % for *M. haemominutum*, whilst also demonstrating that 11 % of specimens were infected with both parasites (Watanabe et al. 2003). In a study undertaken in Saskatchewan and Alberta, PCR was used to detect *M. haemofelis* and *M. haemominutum* in naturally infected cats. Within the group of cats with suspected haemobartonellosis, PCR was positive in 13 of 18 (72 %); 12 of 18 (66 %) were infected with *M. haemofelis*, and 1 of 18 (6 %) with *M. haemominutum*. Eight of the 22 cats (36 %) in the NRA/other infected group had positive PCR results (Kurtdede and Ural 2004). This slight disparity between this reported study and ours may reflect differences in group definitions, sampling methods or sample size. Alternatively, disease prevalence may differ between the geographic areas of the different studies.

A study applying Giemsa staining technique in blood samples of 200 dogs and 112 cats of different ages, breeds and sexes was examined for 1 year in Shiraz, Iran. Two

Table 1 Positive results for demographic traits

Variables	Methods	Group	Sick positive sample (%)	Control positive sample (%)
Age	Direct blood smear	<1 year	13 (41.9)	8 (50)
		1–3 years	2 (40.0)	6 (46.2)
		>3 years	6 (66.7)	10 (62.5)
	PCR	<1 year	18 (58.1)	10 (62.5)
		1–3 years	5 (100)	9 (69.2)
		>3 years	9 (100)	14 (87.5)
Sex	Direct blood smear	Female	10 (35.7)	7 (43.8)
		Male	11 (64.7)	17 (58.6)
	PCR	Female	18 (64.3)	12 (36.4)
		Male	14 (82.4)	21 (36.6)
Breed	Direct blood smear	Nonpedigree	19 (44.2)	23 (54.8)
		Pedigree	2 (100)	1 (33.3)
	PCR	Nonpedigree	30 (69.8)	32 (76.2)
		Pedigree	2 (100)	1 (33.3)
Outdoor access	Direct blood smear	Yes	17 (51.5)	4 (50.0)
		No	4 (33.3)	20 (54.1)
	PCR	Yes	25 (75.8)	4 (50.0)
		No	7 (58.3)	29 (78.4)
Castration	Direct blood smear	Yes	21 (48.8)	18 (62.1)
		No	0 (0.0)	6 (37.5)
	PCR	Yes	30 (69.8)	21 (72.4)
		No	2 (100)	12 (75.0)
Density of living place	Direct blood smear	Group	2 (22.2)	15 (50.0)
		Single	19 (52.8)	9 (60.0)
	PCR	Group	5 (55.6)	24 (80.0)
		Single	27 (75.0)	9 (60.0)

(1.79 %) cats (male, 1–2 years old) were found positive for *H. felis*. The vital signs of both infected cats were normal except for one which showed slight depression and weakness (Jafari Shoorijeh et al. 1999). In our survey, only 50 % of samples were positive in direct blood smears, while 72.22 % of samples were positive using PCR. It should be noted that none of the positive samples on direct blood smear were negative in the PCR assay. The considerable difference in the number of positive cases between the direct blood smear test and PCR in the process of *H. felis* detection can be attributed to various, sometimes unknown, factors and also individual technician error which might lead to false-negative cases in direct blood smear tests.

In Tehran (Iran), *H. felis* was detected in Giemsa-stained blood smear preparations of 23 cats (0.03 L/L); ages ranged from 0.3 to 3 years (median, 1 year). PCV on admission was (0.1–0.43 L/L; median, 0.28 L/L). Twelve out of 23 (PCV; median, 0.25 L/L) had secondary haemobartonellosis, whilst the others had primary haemobartonellosis (PCV; median, 0.33 L/L) (Fathi et al. 2003). Statistical analysis was performed to evaluate associations between haemoplasma infection, age, sex, breed, HCT values and anaemia status. Thirty-four out of 147 Australian cats (23.1 %) were positive for *Candidatus M. haemominutum*, six cats (4.1 %) were positive for *M. haemofelis* and one cat (0.7 %) was positive for both. Old age, male and nonpedigree were also reported as effective factors by these authors (Tasker et al. 2004).

With regard to some of the aforementioned studies (Green 2006; Tasker et al. 2004; Vansteeenhouse et al. 1993; WilliB et al. 2006), old age, not being castrated, access to the outside, high density of cat population and masculinity can all be considered as risk factors and predicting factors of *H. felis* infection, while in our study, with regard to the statistical analysis, only the age of these factors had a significant predicting effect on this infection. Our results are similar to those previously reported by Grindem et al. (1990), which indicated that younger cats are more prone to infection by these microorganisms (Grindem et al. 1990). One reason for this might be the fact that healthy animals had been held near infected ones and then samples were obtained from both groups. This might increase the probability of infection in the young animals which are being held near the infected ones; hence, the role of age in

Table 2 The assessment of the relation between the positive result in direct blood smear and PCR assay by clinical symptoms

Variables	Methods	Chi-square (X^2)	*df*	*P* value
Anorexia and weight loss	Direct blood smear	2.291	1	0.13
	PCR	1.209	1	0.272
Depression	Direct blood smear	0.185	1	0.667
	PCR	0.923	1	0.337
Weakness	Direct blood smear	0.044	1	0.833
	PCR	0.055	1	0.814
Splenomegaly	Direct blood smear	2.045	1	0.153
	PCR	0.787	1	0.375
Pale mucous membranes	Direct blood smear	0.055	1	0.814
	PCR	0.001	1	0.977
CRT increase	Direct blood smear	0.6	1	0.438
	PCR	0.173	1	0.677
Tachycardia	Direct blood smear	1.538	1	0.215
	PCR	10.438	1	0.001
Dehydration	Direct blood smear	3.045	1	0.048
	PCR	15.746	1	0.000
Fever	Direct blood smear	4.406	1	0.036
	PCR	8.633	1	0.003
Icterus	Direct blood smear	0.714	1	0.677
	PCR	4.846	1	0.028

Table 3 Haematological results of positive and negative cases

Variable	Methods	Group	Mean rank (*X*)	SD	*t* test	*P* value
WBC	Direct blood smear	Positive	19,000	9,092.7	−0.708	0.48
		Negative	20,700	13,925.4		
	PCR	Positive	19.700	10,854	0.244	0.808
		Negative	20,300	13,981.4		
RBC	Direct blood smear	Positive	6,070,000	1,360,117	−2.237	0.028
		Negative	6,620,000	939,575		
	PCR	Positive	5,860,000	1,507,272	2.465	0.016
		Negative	6,530,000	1,002,670		
Neutrophil	Direct blood smear	Positive	51.36	12.74	−3.017	0.003
		Negative	60.78	16.63		
	PCR	Positive	54	13.083	−2.081	0.04
		Negative	61.44	18.34		
Lymphocyte	Direct blood smear	Positive	46.87	12.39	2.511	0.014
		Negative	38.24	19.41		
	PCR	Positive	44.17	13.55	1.48	0.142
		Negative	38.36	22.96		
Platelets	Direct blood smear	Positive	342,000	212,250.9	0.454	0.652
		Negative	314,000	217,658.4		
	PCR	Positive	337,000	211,964.08	0.55	0.585
		Negative	301,000	222,530		
Hematocrit	Direct blood smear	Positive	33.01	7.26	2.117	0.037
		Negative	35.95	5.82		
	PCR	Positive	31.3	7.69	2.905	0.005
		Negative	35.71	5.91		
Reticulocyte	Direct blood smear	Positive	1.03	0.22	3.014	0.003
		Negative	0.89	0.202		
	PCR	Positive	0.99	0.22	2.238	0.028
		Negative	0.88	0.21		

the spread of infection is manipulated. Castration age in Iran is also not in complete accordance with the international standards, and most of the cats are castrated after the age of puberty, while, due to territory conflicts and pair bonding, it is highly probable that the animal might have already been infected with *H. felis* during this period. It is also possible that the animal might get infected during the later years of their life. As for free access to the outside, according to the common customs in Iran, most cat owners allow the animal to have access to the outside; therefore, this factor, which is applied to most cats in our study, might not be specifically predictive of *H. felis* infection.

Kurtdede and Ural (2004) described four cases of feline haemobartonellosis in which anaemia and high fever had been the predominant presenting symptoms. The abnormalities found on routine haematological examination included mild normocytic–normochromic regenerative anaemia, normocytic–hypochromic and macrocytic–normochromic anaemia, mild eosinophilia (13 % in cat 1 and 11 % in cat 4) and monocytosis (6 % in cat 1 and 12.5 % in cat 3) (Kurtdede and Ural 2004). However, in our study, there were statistically significant differences ($P<0.05$) between control and infected groups regarding the quantitative variables including fever and dehydration in direct blood smear, and tachycardia, icterus, fever and dehydration in PCR, that can be explained by the effects of the parasites on blood cells and the simulation of the immune system (Table 2).

Cats with normal CBCs that were PCR positive were infected with *M. haemominutum* and may have been latent carriers of this organism. These results are consistent with previous reports that *M. haemominutum* is less pathogenic than *M. haemofelis* (Green 2006; Jensen et al. 2001; Westfall et al. 2001). Moreover, on direct blood smear exam, haematological factors including erythrocytes, neutrophils, lymphocytes, HCT and reticulocytes were significantly different between control and infected groups. The results obtained for all haematological factors in PCR assay were the same as those in direct blood smear, except for lymphocyte count (Table 3). The reduction of HCT and RBC in infected cats in both test methods can be explained through the effects of microorganisms on erythrocytes.

To our knowledge, this study is the first to evaluate the prevalence of *M. haemofelis* and *M. haemominutum* in cats in Esfahan (Iran) using the PCR technique. The results of this study indicate that *M. haemofelis* and *M. haemominutum* have a high prevalence in central parts of Iran. Cats are therefore important sources of *Haemobartonella* infection in this area. Finally, by comparison of the results obtained for *H. felis* infection through the two methods of direct blood smear and PCR, statistically significant differences were observed, based on which the reliability and validity of PCR assay are illustrated. PCR assay can be considered a valuable diagnostic tool or screening test for the detection of haemobartonellosis. Also, it can be concluded that creating haematological profiles for every animal referred to a clinic can be the best way of evaluating preclinical changes and early diagnosis of different diseases.

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