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Hemotropic Mycoplasmas in Stray Cats in Kerman, Iran

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ABSTRACT

***Background*:** Feline haemotropic mycoplasma are a group of pleomorphic bacteria causing hemolytic anemia along with anorexia, lethargy, dehydration, weight loss and in many cases sudden death in infected animal. However, there is a limited data on the prevalence of these organisms in Iranian cats.

***Methods*:** We investigated the presence of feline haemotropic mycoplasma and probable risk factors for these infections among 60 ectoparasite-infested stray cats in southeast of Iran using PCR assay.

***Results*:** The overall prevalence of haemotropic mycoplasma was estimated 18.3%. Pallor mucous membrane, anorexia, weight loss and splenomegaly were the most common signs and the infection rate was significantly higher in symptomatic cats in comparison with apparently healthy ones (P = 0.001). Age, gender and hematological alterations were not significantly associated with infection status while the level of BUN, creatinine, total protein and globulin were significantly higher among infected animals.

***Conclusion*:** The prevalence of feline hemoplasma infection in stray cats seems to be considerable in our study. More investigations are needed to obtain further information on epidemiological aspects of hemoplasmas in cats in Iran.

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Introduction

Haemotropic mycoplasmas, also known as hemoplasmas, are the responsible agents for acute or chronic infectious anemia in cats, dogs and other vertebrate species (1, 2). This cell wall-less pleomorphic bacteria attach to the surface of red blood cells (RBCs) and induce hemolytic anemia by extravascular destruction and intravascular lysis of erythrocytes (3). Acute hemolysis caused by hemoplasmas could be associated with anorexia, lethargy, dehydration, weight loss and in many cases sudden death of infected animal (1, 2). Moreover, animals recovered from infection may remain carriers of the agents with no external signs (3). *Mycoplasma haemofelis* (Mhf), Candidatus *Mycoplasma haemominutum* (CMhm) and most recently Candidatus *Mycoplasma turicensis* (CMt) are the three recognized species of feline haemoplasmas. Mhf is known to be the most pathogenic species often inducing severe anemia and clinical signs of disease (4, 5), however all species have been reported and discussed in many epidemiological studies (6-10). Human infections with hemoplasmas have also been reported in a number of investigations (11, 12).

Hemoplasmas are non-culturable in vitro. Hence, the valid protein-based serological assays would not be applicable to them (2) which raises a challenge in investigating these species in more detail. Prior to the advent of polymerase chain reaction (PCR) testing, microscopic examination of blood smears was the only way to identify hemoplasmas (13) which did not seem to be flawless since the presence of artifacts resulting from staining and Howell-Jolly bodies in red blood cells could lead to false-positive results. Besides, despite severe infection during acute phase, false-negative results could occasionally occur due to the rapid clearance of pathogens by the spleen and the cyclic recurrence of microorganisms in erythrocytes (1, 4).

The development of PCR technology has opened up the door to a much better understanding of the characteristics and mechanism of hemoplasmas and allowed more precise investigations on them. Infectious anemia or hemoplasmosis has been globally diagnosed in pet cats and wild felids by using PCR-based techniques (2). A considerable number of research studies have so far addressed the amplification of 16S RNA gene sequences for *Mycoplasma* spp. (14-16). Yet, the epidemiology of hemotropic mycoplasmas is still poorly understood since there are no reports available on molecular characterization of these pathogens in many countries (1, 17). In Iran, only one investigation in Tehran city was carried out in this field and prevalence of hemotropic mycoplasmas in owned cats was reported to be 22% (18). There is no available data on the infection status of stray cats. This study aimed to investigate the prevalence of hemotropic mycoplasmas in stray cats in southeast of Iran using PCR method.

Material and method

Study area

The study was done in Kerman city; located on a high margin of Lut Desert in the southeast of Iran. This area is arid, with hot summers and violent sand storms in spring whereas its climate is relatively cold in other seasons.

Animals

One hundred forty two stray cats were gathered by a double door live trap cages contain baits by a volunteer cat rescue group in a Trap-Neuter-Return program. The cages were hold in five different parts of Kerman city near garbage dumpsters. These cats were randomly selected with no limitation for age, sex, and clinical status during September 2013 to April 2014 for our study. Before contraception surgery, all animals were clinically monitored for three consecutive days and a detailed questionnaire was filled out for each animal, with data on age, sex, observations of ectoparasites and clinical status. Sixty stray cats with ectoparasite infestation (tick and flea) were

selected as a high risk population for this study. Based on the most common clinical signs reported in cats infected with hemotropic mycoplasmas, the diagnostic criteria were delineated. The clinical signs included anorexia, cachexia, tachycardia, mucous membranes pallor, icterus, jaundice and splenomegaly (4). Any cats having at least two clinical signs was considered symptomatic and those without any obvious clinical sign or symptom were placed in the apparently healthy group.

Hematology and biochemistry evaluation

Blood samples (5 ml) were drawn from the jugular vein aseptically. One milliliter was immediately inserted into an anticoagulant containing tube (ethylene diamine tetra-acetic acid) for hematological evaluation. Complete blood counts were manually made for each animal and the presence of hematological disorders such as anemia (Heamatocrite <20), leucopenia or leucocytosis (Less than 5500 to more than 19500 leukocyte/µl of blood), thrombocytopenia (<1500000//µL) and changes in differential leukocyte count was recorded.

Three milliliter of blood was put into plain tubes. Serum samples were separated by centrifugation at 3,000 rpm for 3–5 min and stored at -20 °C for biochemical examination. Total protein, globulin, blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by an autoanalyser (Auto lab, AMS -18A, China). The rest of blood samples were placed into DNase, RNase Eppendorf tubes and kept frozen at -20 °C for DNA extraction.

DNA extraction

For DNA extraction from blood samples, we applied the viral gene-spin kits (VeTeK™, South Korea). The extracted DNA was then stored at -20 °C until PCR analysis was done.

PCR assay

PCR was performed for all samples by VeTeK™ HBN-F rapid PCR kit (VeTeK™, South Korea) according to company instructions. The assay was able to detect *Candidatus* M. haemominutum and/or *M. haemofelis* by detecting 322-bp fragment of the 16S rRNA gene of *Mycoplasma* spp. in genomic DNA samples. Amplified DNA was subjected to electrophoresis on a 1.5% agarose gel for 40 min at 120 V, pre-stained with ethidium-bromide and viewed under ultra-violet light.

Statistical analyzes

In Statistical analysis (SPSS version 18), positive PCR test was set as an outcome variable and the independent variables were sex, age, health status (symptomatic, apparently healthy), hematological and biochemical alterations. The effect of independent variables on the outcome variables were evaluated by chi-square and Fisher's exact test and Odds Ratio (OR) calculation. Differences were considered significant if the P value was <0.05.

Results

Based on PCR assay, a total of 11 (18.3%) of all samples yielded positive results for hemotropic mycoplasma.

Case characteristics of studied cats are shown in table 1. Twenty one cats were symptomatic. The most common signs in clinical examinations were pallor mucous membrane, anorexia, weight loss and splenomegaly (Table 2). The prevalence of hemoplasma infection in the symptomatic cats was significantly (P = 0.001) higher in compare with asymptomatic groups but there was no relationship between age and positive PCR result. PCR status has also showed no significant association with animals' gender.

Table 1. Age, gender and health status of hemoplasma-negative and -positive cats.

Variables	No. of PCR		Odds ratio	95% CI	*P*
	Negative (%)	Positive (%)			
Age (yr)					
≤3	37 (75.5)	8 (72.7)	1.0		
>3	12 (25.5)	3 (27.3)	1.2	0.26-5.1	0.8
Gender					
Female	27 (55.1)	6 (54.5)	1.0		
Male	22 (44.9)	5 (45.5)	1.02	0.27-3.8	0.97
Health Status					
Symptomatic	10 (20.5)	10 (90.9)	35.5		
Apparently healthy	39 (79.5)	1 (9.1)	7.81?	4 -1576.	0.001

Table 2. Frequency of clinical signs among hemoplasma infected cats.

Cat No	Sex[a]	Age[b]	Health status
2	F	6Y	Anorexia, weight loss , pale mucus membrane
9	M	9M	Clinically healthy
20	F	2Y	Bite wounds, pale mucus membrane
25	F	3Y	Tachycardia, pale mucus membrane
28	M	2Y	Pale mucus membrane, splenomegaly
32	*M*	4 Y	Pale mucus membrane, vomiting, splenomegaly
39	F	2 Y	Bite wounds, diarrhea, anorexia, weight loss
47	F	2Y	Pale mucus membrane, anorexia, weight loss
51	M	4Y	Bite wounds, pale mucus membrane, anorexia, weight loss
58	F	2Y	Pale mucus membrane, splenomegaly
59	M	1Y	Diarrhea, anorexia, weight loss
Total	-	-	*Clinically healthy: 1* *Symptomatic: 10*

a: sM=Male F=Female b: Y=Year M=Month

Hematologic and biochemical blood parameters of PCR-positive and negative animals are summarized in table 3. Based on our findings, no significant alteration was seen in hematocrit level and log of red blood cell and white blood cell count in hemoplasma infected cats. On the other hand, Bilirubin, AST and ALT level was in normal range, however level of BUN, creatinine, total protein and globulin were significantly higher in hemoplama-infected cats. The disparity of results may be attributed to the methods used for detecting resistance among the isolates and their different degrees of accuracy, making molecular PCR techniques imperative to the identification of this gene.

Table 3. Selected hematologic and biochemical blood parameters of hemoplasma-negative and -positive cats.

Variables	PCR negative 49 cats			PCR positive 11 cats			Reference range (19, 20)	*P*
	Mean	Std. Dev.	95% CI	Mean	Std. Dev.	95% CI		
PCV[a] (%)	27.32	7.04	25.30-29.35	26.09	6.62	21.64-30.54	20-45	0.5975
Log RBC[b] /µl	6.54	0.16	6.49-6.58	6.53	0.28	6.34-6.73	6.77-7	0.9442
Log WBC[c] /µl	3.77	0.16	3.72-3.82	3.67	0.10	3.60- 3.74	3.74-4.29	0.0639
BUN[d] (mg/dl)	23.08	6.46	21.22-24.93	36.90	15.10	26.76-47.05	14-35	<0.001
Creatinine (mg/dl)	1.54	0.49	1.39-1.68	2.33	1.10	1.59-3.07	0.6– 2.4	<0.001
Total protein (mg/dl)	6.51	0.90	6.25-6.77	8.28	1.54	7.24-9.32	5-8.3	<0.001
Globulin (mg/dl)	3.13	1.01	2.84-3.42	4.31	1.71	3.16-5.46	2.4-5.1	0.003
Total bilirubin (mg/dl)	0.07	0.06	0.05-0.09	0.09	0.11	0.01-0.17	0.1-0,4	0.5
ALT[e] (u/l)	59.83	24.51	52.79-66.87	49.18	19.11	36.33-62.02	8.3-55	0.1826
AST[f] (u/l)	22.77	16.26	18.10-27.44	19.00	7.65	13.85-24.14	0-50	0.4578

Parameters are listed in SI units. Abbreviations are: a: PCV= Packed cell volume, b:RBC= Red blood cell, c:WBC= White blood cells, d:BUN= Blood urea nitrogen, e:ALT= Alanine aminotransferase, f:AST= Aspartate aminotransferase.

Discussion

Several investigations have so far been carried out to assess the prevalence of feline hemotropic Mycoplasma infection. While *Candidatus* M. turicensis has only been reported in Switzerland, South Africa, Australia, USA and the subsequent studies in Italy (1921), Willi *et al*. have shown that the two more common species of *Hemoplasmas*, *M. haemofelis* and *Candidatus* M. haemominutum are globally distributed and co-infection with 2 to 3 species may occur in some cases (2). This is the second molecular study reporting the prevalence of feline hemoplasma with associated hematological and biochemical data in cats in Iran. The first study was conducted on owned cats in Tehran and estimated prevalence was 22% (18). However, our study indicated that haemotropic mycoplasma spp. have a nearly same prevalence (18.3%) in stray cats in Iran. *M. haemofelis* was the most prevalent species in the Ghazi Saeed et al study in Iran but unfortunately mycoplasma species were not differentiated by our commercial PCR kit.

Haemotropic mycoplasma infected cats were significantly anemic based on decreased hematological variables in the study of Ghazi Saeed et al (18) and Tasker *et al.* on Australian cats (8). These findings are in contrast to our data and Willi et al (9). These differences may be

related to the various prevalence of different species in geographical areas. Using PCR assays, in 2001 in United States, around 30% of anemic cats were found to be infected with haemotropic mycoplasmas showing a dominance of *M. haemofelis* individually or in combination with *Candidatus M. haemominutum*. In addition, PCR showed that about 14% of healthy cats (without any clinical and laboratory features) were infected with haemotropic mycoplasmas (15). In the present study, 50% of diseased cats and 2.5% of healthy ones tested PCR positive for hemoplasmas which showed that clinicians must certainly consider the risk of hemoplasma infection in diseased cats in our country.

The higher prevalence of hemoplasma infections in older male cats have been reported so far in a few studies (9, 18, 19, 20, 21, s22), whereas we found no association between gender and presence of infection in cats. In spite of that, in agreement with Willi's study (9), association between hemoplasma infection and elevated BUN, creatinine and total protein and globulinprotein level was noted in our study. This association could be causal but elevation of total protein and globulin was a common finding in chronic infection disease and elevated BUN, creatinine could also be accounted for older age of infected cats which predispose them to chronic renal failure (4).

In a similar study in Italy, Gentilini *et al*. investigated the epidemiological and hematological features of three common species of hemoplasmas. The overall prevalence of hemoplasma infections in Italian cats was similar to our study. Infection with *M. haemofelis* was found to be associated with decrease of hematocrit level in the Italian cats. Moreover, RBC values of infected cats were significantly lower than uninfected cats. WBC values evaluation also showed an increasing trend in infected animals. But we found no association between RBC and WBC levels and infection (1921).

Sykes *et al.* performed a Real-time PCR assay in American cats in 2008. A total of 310 cats with cytological evidence of hemoplasmas infection were selected in their study and considerable differences in the results of cytological and molecular investigations were reported (2123). A case-control study on hematological changes, prediction and treatment of hemoplasmas infected cats in Iran was carried out by cytological method which reported very low prevalence (3%) of disease (22, 24). While the prevalence of infection in Iranian owned cats were estimates 22% in the following investigations by PCR method (18) which is approximately same to the present study. Regarding to these studies PCR could be even used as a screening method for feline hemoplasmas infection.

Conclusion

In this study, for the first time the existence of feline hemoplasma infection in stray cats was noted in Iran. Most of the owned cats in old cities and small towns of Iran were grown up in unconfined circumstances with open access to outdoor and stray cat population because their owners mostly live in houses with courtyard and garden. This raising type could create a considerable risk of disease transmission between these two populations via direct contact or ectoparasite transmission. Further investigations, including species specific PCR studies on stray and owned cats in our country are needed to obtain more information on epidemiological aspects of haemotropic mycoplasmas in Iranian cats.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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