SAĞLIK BİLİMLERİ DERGİSİ
JOURNAL OF HEALTH SCIENCES
Erciyes Üniversitesi Sağlık Bilimleri Enstitüsü Yayın Organıdır

Araştırma Yazısı 2015; 24: 1-6

DIAGNOSIS of *MYCOPLASMA HAEMOFELIS* and CANDIDATUS *MYCOPLASMA HAEMOMINUTUM* USING PCR ASSAY in CATS*

KEDİLERDE PCR İLE *MYCOPLASMA HAEMOFELIS* VE CANDIDATUS *MYCOPLASMA HAEMOMINUTUM*'UN TANISI

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ABSTRACT

In this study, it was aimed to determine haemoplasmosis using polymerase chain reaction (PCR) analysis in cats. The material for this study consisted of blood samples collected from 84 cats of aged average 5.5 years on average (6 months-10 years) and belonged to different strains and sexes (41 females and 43 males). Blood samples were analyzed both cytologically and with PCR. The PCR was performed with the specific primer pairs in order to amplify 170 bp and 193 bp region of 16S rRNA gene from *Mycoplasma haemofelis* and Candidatus *Mycoplasma haemominutum*, respectively. All positive samples were sequenced in both directions with the amplification primers. The PCR analysis showed that 8 of 84 cats (9.52%) were haemoplasma positive and 4 cats (4.76%) were infected with *M. haemofelis*, 3 (3.57%) were infected with Candidatus *M. haemominutum* and one cat (1.19%) was co-infected. To the best of the authors's knowledge, this study reports the first molecular characterization of *M. haemofelis* and a co-infection with *M. haemofelis* and Candidatus *M. haemominutum* in cats in Turkey.

Key words: cat, cytological analysis, haemoplasmosis, PCR

ÖZET

Bu çalışmanın amacı, kedilerde polimeraz zincir reaksiyonu (PCR) ile haemoplasmozisin belirlenmesidir. Çalışmanın materyalini farklı ırk ve cinsiyette (41 dişi ve 43 erkek), ortalama 5.5 yaşlarında (6 ay-10 yaş) 84 kediden toplanan kan örnekleri oluşturdu. Kan örnekleri hem sitolojik, hem de PCR yöntemi ile incelendi. PCR reaksiyonu için *Mycoplasma haemofelis* ve Candidatus *Mycoplasma haemominutum*'un 16S rRNA gen bölgelerinden sırasıyla 170 bp and 193 bp bölgesini çoğaltmak için spesifik primerler kullanıldı. PCR analizi sonucunda 84 kedinin 8'i (%9.52) haemoplazma pozitif olarak belirlendi ve bunlardan 4'ü (%4.76) *M. haemofelis*, 3'ü (%3.57) Candidatus *M. haemominutum* ve bir kedi (%1.19) her iki etken açısından pozitif bulundu. Bilgilerimize göre bu çalışma ile Türkiye'de kedilerde *M. haemofelis*'in ilk moleküler teşhisi yapılmış ve *M. haemofelis* ve Candidatus *M. haemominutum* enfeksiyonlarının birlikte tespiti ilk kez bildirilmiştir.

Anahtar kelimeler: haemoplasmozis, kedi, PCR, sitolojik analiz

INTRODUCTION

Haemotropic mycoplasmas attaching to the host's erythrocytes surface are unculturable, gram-negative, obligate and wall-less bacteria which are known as the causative agents of infectious anemia in a wide variety of mammals including feline (1-3).

Feline infectious anemia (FIA), a disease of cats, is caused by three mycoplasma species, namely, *Mycoplasma haemofelis (*Mhf*), "*Candidatus *Mycoplasma haemominutum" (*CMhm*)* and "Candidatus *Mycoplasma turicensis*" (CMt) (4,5). Among the three mycoplasma species *M. haemofelis* has been reported as the most pathogenic feline hemoplasma (6,7). In addition, a recent species, "Candidatus *M. haematoparvum*", was reported in a canine haemoplasma in two cats in the USA (8).

The pathogens can be visualized as dark purple-blue coccoids, rings or short chains on the erythrocyte surface, using Romanowsky-type stain such as Giemsa, Wrigth Giemsa or DiffQuick of blood smears (1-3,9). Polymerase chain reaction assays, based on the 16S rRNA gene are also used to diagnose feline haemoplasma infections for being more sensitive and specific methods than cytology (2).

*This study was supported by funding from Erciyes University (Project number: TSY-12-4066)

Makale Geliş Tarihi : 02.10.2014
Makale Kabul Tarihi: 03.02.2015

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Recently, Haemotropic mycoplasmas have been reported as potential zoonotic pathogens in mammals (9). For instance, dos Santos et al (10), found a *Mycoplasma haemofelis*–like infection in an HIV-positive patient in Brazil. In addition, a hemolysis associated with haemoplasma infection (11), and two variants of an organism resembling ovine hemoplasma in human (12) were also reported.

There have been little or no emphases on *Mycoplasma haemofelis* whereas a few studies have reported Candidatus *Mycoplasma haemominutum,* using molecular diagnostic methods previously in Turkey (13, 14). To the best of authors' knowledge, this study represents the first report of *Mycoplasma haemofelis* infection and co-infection with *Mycoplasma haemofelis* and Candidatus *Mycoplasma haemominutum* in Turkey.

MATERIALS AND METHODS

Blood samples were collected from 84 cats of which 40 cats were admitted to Faculty of Veterinary Clinics at Erciyes University between 2012 and 2013 years; and 44 cats were selected from a Cat Shelter of Kayseri Metropolitan Municipality. The cats were average 5.5 years old (6 months-10 years) and belonged to different strains and sexes (41 females and 43 males). Breed, age, sex, outdoor access, density of living place, vaccinations, the presence of ectoparasites, reasons for admission to the clinics of all cats were recorded.

In total, 2.5 ml blood samples were taken from vena cephalica antebrachii with EDTA tubes for thin blood smear, hematological examinations and PCR analysis. All blood samples were stored at -20ºC for PCR analysis after blood smear and hematological examinations.

Direct blood smears and hematology

Blood smear was performed, using a drop of blood on a microscope slide and staining it with Giemsa methods. The samples were evaluated for visualisation of the haemotropic mycoplasma microscopically. Complete blood counts were examined by electronic cell counter (Mindray BC-2800 Vet®, China, at Erciyes University, Faculty of Veterinary Medicine Clinics).

DNA extraction and PCR Assay

DNA was obtained from 200 µl of blood using a genomic DNA purification kit (Purelink Genomic DNA Mini Kit, Invitrogen®, USA) according to manufacturer's protocol.

Primers, previously described by Jensen et al. (15) for PCR reaction that target the 16S rRNA gene (5'- ACG AAA GTC TGA TGG AGC AAT A-3' forward primer and 5'- ACG CCC AAT AAA TCC GRA TAA T-3' reverse primers (Novagentek®, Ankara)) were used producing a170 bp and a 193 bp amplicon for Mhf and CMhm, respectively.

PCR reaction contained 5 µl DNA, 3,5 mM $MgCl_2$, 2 U Taq polimerase, 50 µM dNTP Mix (Fermentas®,Lithuania) and 0.2 µM each primer, made up to final volume 25 µl with sterile ultrapure water. Next, PCR reaction conditions were applied to the initial denaturation step for 4 min at 94º C, followed by 35 cycles of 1 min denaturation at 94º C, 30 sec. primer annealing step at 60º C, and 30 sec. extension at 72º C. In the last cycle, the extension was hold at 72º C for 10 min. (15,16). Thermal cycler (MyGenie96 Thermal Block®, South Korea) was used for PCR analyses. *M. haemofelis* and Candidatus *M. haemominutum* DNAs (gifts from Dr. Severine Tasker, Bristol University, Department of Small Animal Medicine) were used as positive controls. In addition, a negative control (sterile water) was included in each PCR run. Reaction products were electrophoresed through 2% agars' gels stained with ethidium bromide and visualized by UVP gel documentation system (Gel Logic 200 Imaging System®, Kodak). *M. haemofelis* and Candidatus *M. haemominutum* were diagnosed by comparing the PCR product size with the size of known positive control DNAs, negative control and a 100 bp DNA ladder.

Statistical Analyses

The data were analyzed using SPSS version 12.00 software (SPSS Inc., Chicago, IL) and expressed as arithmetic mean, standard deviation and percentage. The relationships between the sex, age, outdoors with haemoplasma positive and haemoplasma negative cats using Chi-square, Fischer exact test were calculated. For comparison of the haematological parameters, Student-t test and for control of normal disturbance of data, Kolmogorov-Smirnov test were used. Statistical significance was defined as $p<0.05$.

RESULTS

Clinical examination results revealed different symptoms such as diarrhea, ecto- and endoparasites, respiratory tract infection, urinary tract infection, tumor, trauma, fracture, icterus, ascites and anemia. Twenty one cats in this study had no contact with other cats and nor had they outdoor access.

Haemotropic mycoplasmas were seen in dark purple-blue coccoids and short chains on the erythrocyte surface. Howell-Jolly bodies were also found at the examination of peripheral blood smear stained with Giemsa (Figure 1).

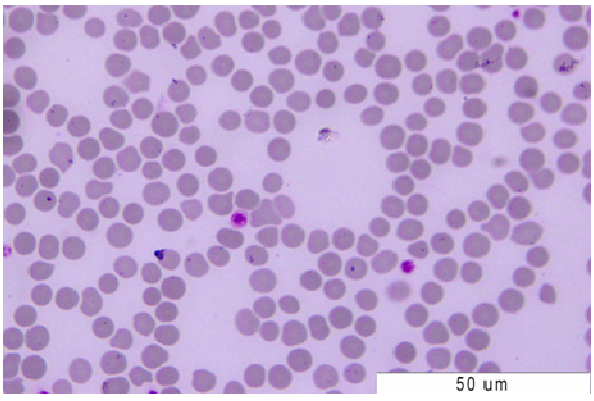

Figure 1. Blood films showing *Mycoplasma* spp. on erytrocyte surface (black arrow). Howell-jolly body (white arrow). Giemsa Stain X100

The feline haemoplasma PCR assays based on the 16S rRNA gene showed that 8 of 84 cats (9.52%) were haemoplasma positive; 4 of which (4/8; 4,76%) were infected with *M. haemofelis*, 3 of which (3/8; 3.57%) were infected with Candidatus *M. haemominutum,* and one cat (1/8; 1.19%) was co-infected. Among the 8

positive samples, 6 cats (7.14%) were collected from Kayseri Metropolitan Municipality Cat Shelter whilst 2 cats (2.39%) were sampled from The Faculty of Veterinary Clinics at Erciyes University.

M. haemofelis (170bp) and *CMhm* (193bp) were differentiated based on the amplicon size following running PCR products at gel electrophoresis (Figure 2).

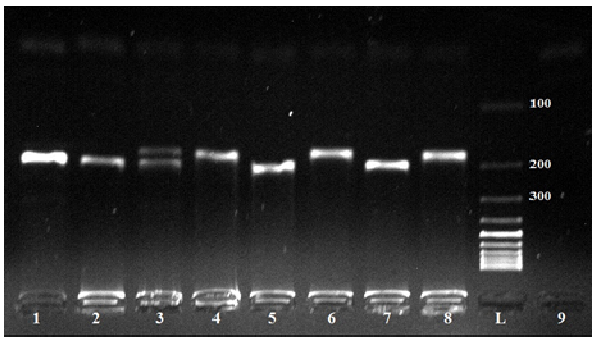

Figure 2. Ethidium bromide-satained agarose gel (2%) eletrophoretogram showing amplified DNA from *M. haemofelis* (170bp) and Candidatus *Mycoplasma haemominutum* (193 bp) from peripheral blood of cats. Lanes are as follows: L. 100 base pair DNA ladder; 1,4,6- *M. haemofelis* (170bp) positive samples; 2,5- Candidatus *Mycoplasma haemominutum* positive samples ; 3. Co-infected *M. haemofelis* and Candidatus *Mycoplasma haemominutum* sample; 7- *M. haemofelis* positive control, 8- Candidatus *Mycoplasma haemominutum* positive control, 9- Negative control (steril ultrapure water)

The clinical examination of haemoplasma positive cats showed that two cats had respiratory system infection, one cat had diarrhea, one cat had endo-parasite infestation, one cat had oedema at low extremity and 5 cats had high fever, whereas no clinical sings were observed in other cats.

The haemoplasma positive cats were at average 3.5 years old (2-5 years old). There was no significant difference between haemoplasma positive and haemoplasma negative cats for sex, age and outdoor access (Table 1) (p> 0.05).

As for the odds ratio, haemoplasma incidence was determined 1.145 and 2.082 times more in outdoor access and ≥4 years-old cats, respectively. In addition, the differences in levels of hemoglobin and PCV were significant (p<0.05). No statistical significance was observed for leukocyte counts between two groups (p>0.05), but five haemoplasma positive cats had leukocytosis (Table 2).

DISCUSSION

Eperythrocytic and unculturable haemoplasmas, also known as *Haemobartonella* and *Eperythrozoon,* have the ability to cause seriously haemolytic anemia. Therefore *Haemobartonellozis* in cats is named as feline infectious anemia. Pathogens can be seen on erythrocyte surface as pink, purple, brawn colored and round, ring shaped or chain formed in the peripheral blood by Romanovsky-type staining such as Giemsa, Wright or DiffQuik stain (18,19). The findings of the current study showed that the organisms were seen as purple-blue coccoids and short chains on the erythrocyte surface at cytological examination similar to the studies conducted. However, this method has poor sensitivity for agent diagnosis in cats with chronic infection and low parasite load or cyclic parasitemia. Besides, cytology may result in false positive diagnosis due to the resembling stain artifacts and Howell-Jolly bodies. Furthermore, differentiation of haemotropic species is difficult with light microscopy. Consequently, in this study, a more reliable and sensitive PCR assay was used (15,16). Haemoplasmas are quite common and infect various mammalian species in the world (9). Recently, the importance of haemoplasma infections has increased due to studies reporting haemotropic mycoplasmas in people with anemia (11,12). Many studies have also investigated epidemiology, prevalence and appropriate therapeutic protocols of haemoplasma infections in cats (3,6,20-23). Tanahara et al. (24), reported that male, middle age and old cats with FIV-infection are prone to haemoplasma. Grindem et al. (21) determined that anemia, FeLV infection, lack of immunisation, the presence of anemia and/or cat bite abscesses, cats younger than 3 years old and outdoors cats carried more risk to haemoplasma infection, whereas gender, race, number of cats in the same household or the presence of fleas were not important factors as much. Similarly, Torkan et al. (3), emphasized that the factors such as castration, gender, outdoors and the number of cats in population were not important. However, previous studies in Swiss (4) and Australian cats (25) reported that haemoplasma infection risk could more likely to occur in older cats. In this study, it was determined that haemoplasma

Table 1. Risk factors of haemoplasma infection in cats in Kayseri province, Turkey

Factor	Modality	Haemoplasma (-) (n, %)	Haemoplasma (+) (n, %)	Fisher's Exact Test
Sex	**Female**	37 (90.2%)	4 (9.8%)	
	Male	39 (90.7%)	4 (9.3%)	0.944
Age (years)	**0-3**	59 (92.2%)	5 (7.8%)	
	≥4	17 (85.0%)	3 (15.0%)	0.38
Lifestyle	**Indoors**	21 (100%)	0 (0.0%)	
	Outdoors	55(87.3%)	8 (12.7%)	0.192

Table 2. Haematological values in haemoplasma positive and negative $\bar{X} \pm S_{\bar{X}}$ **cats** ( )

Parameters	Referance Range (17) (minimum-maximum)	Haemoplasma (-)	Haemoplasma (+)	Statistical Significance**
WBC (10^3/µl)	5.5-19.5	17.47±1.12	19.49±2.54*	*p=0.571*
RBC (10^6/µl)	4.6-10.0	7.95±0.21	12.23±5.00*	*p=0.421*
HGB (g/dL)	9.3-15.3	12.56±0.36	11.24±0.34	*p=0.012*
PCV (%)	28.0-49.0	38.19±1.02	35.29±0.85	*p=0.035*
MCV (fL)	39.0-52.0	48.19±0.41	50.21±1.17	*p=0.130*
MCH (pg)	13.0-21.0	15.74±0.15	15.91±0.29	*p=0.705*
MCHC (g/dL)	30.0-38.0	32.40±0.32	31.78±0.37	*p=0.531*
PLT (10^3/µl)	100-514	227.86±16.04	253.88±49.74	*p=0.618*
Eosinophil (%)	2-12	6.70±0.88	5.24±1.46	*p=0.599*

***: Different according to the standard value**

****:Statistically significance control of mean values between haemoplasma negative and haemoplasma positive cats**

infection can be seen as much in four years age and older cats and cats with outdoor access than other; but, gender is not an important factor for haemoplasmosis as much. This is supported by previous studies (1,2,4,22,25-27) where haemoplasma infection was found to be significantly related to older age. The increasing odds ratio of haemoplasma infection in older ages may be due to the cumulative effect of exposure to the pathogen over time. In particular, as the positive blood samples (7.14%) and co-infection (1.19 %) was high among the cats from a Cat Shelter in Kayseri Metropolitan Municipality, it is argued that the infection risk could be increased depending on the increase in the number of cats. The increased risk of infection was considered to be due to the transmission of infection via cat bites and fighting between cats with increased cat number in population.

The clinical symptoms of haemoplasmosis vary depending on co-factors such as the presence of immunsuppression, retrovirus infection and the cycle of infection, species and the strain of hemoplasma (28). Typical clinical sings of acute Mhf infection include pale mucosa, cardiac murmur, lethargy, weakness, tachycardia, dispnea, tachypnoea, hepato-splenomegaly, lymphadenopathy, depression, dehydration, pica and weight loss. Icterus is rarely observed unless severe acute hemolysis develops. Fever is a common clinical finding; especially in cats with mature immune system may be only prominent clinical symptom. Hypothermia may occur when cat are about to die. Anemia may not be determined in cats with chronic haemoplasma infection. As there is no significant difference for haemoplasma prevalence between anemic and nonanemic cats (2,4,25), these symptoms are not pathognomonic for haemoplasma infections (1). In this study, the infection may have become chronic as there were no clinical sings except for fever in five haemoplasma positive cats using PCR assays and findings HGB and PVC parameters were determined in reference limits between haemoplasma positive and negative cats at haemotological examinations, they were statistically significant. Besides, these cats may be carrier of haemoplasma infection due to the hematocrit level of 25-35% in carrier cats (18) and the clinical symptoms may occur at co-infection with immunosuppressive agent (e.g. retrovirus) in carrier cats.

Studies investigating the haemoplasma prevalence, using PCR in the world vary, depending on the geographic region. For instance the prevalence of haemoplasma in blood samples of 1585 cats in UK was reported as Mhf 2.8%, CMhm 11.2% and CMt 1.7% (29). Willi et al. (5) the prevalence of haemoplasma was determined as Mhf 4.8 %, CMhm 24.0% and CMt 10.0% in Australia, Mhf 1.6 %, CMhm 17.0% and CMt 2.3% in UK and Mhf 15.0%, CMhm 38.0 % and CMt 26.0% in South Africa. Also, Mhf 1.5%, CMhm 10.0% and CMt 1.3% in Switzerland was reported (4). Fujihara et al. (26) in Japan determined Mhf 21.0%, CMhm 47.0% and CMt 10.0%. In the study of stray 45 cats in Ontario positive infections for Mhf 47.0% ve CMhm 13.0% were found (27). Studies concerning haemoplasma prevalence using both cytological examination and PCR have also been in Turkey (13,14,30-32). Haemoplasma infection rate was reported as 20.0% in Ankara (31) and 14.9% in Van (32) by cytological examination. Ural et al. (14) studied haemoplasma incidence, using PCR assay in 4 Turkish provinces (Ankara, Antalya, Bursa, İzmir) and found the haemoplasma incidence as 18.9% while only determined Candidatus *M. haemominutum*. In the present study, PCR assays of feline haemoplasma showed that 8 of 84 cats (9.52%) were haemoplasma positive; 4 of which (4/8; 4.76%) were infected with *M. haemofelis*, 3 of which (3/8; 3.57%) were infected with Candidatus *M. haemominutum,* and one cat (1/8; 1.19%) was co-infected. The lower infection rate in this study compared to another study in Turkey may reflect differences in sampling methods (included cats), sample size or diagnosis techniques. The study conducted in Ankara (31) included only cats with anemia using cytology. The study in Van (32) included only cats from Van Cats Shelter using cytology and Ural et al. (14) selected cats showing clinical signs of infection and more male cats.

In this study, the feline infectious anemia incidence was

determined as 9.52% based on PCR assays in Kayseri Province. To the best of authors' knowledge, this study reported the first molecular characterisation of *M. haemofelis* and a co-infection with *M. haemofelis* and Candidatus *Mycoplasma haemominutum* in a cat in Turkey. Four years old and older cats and outdoors cats appeared to be common risk factors for feline haemoplasma infection. Besides, the typical clinical symptoms were not observed at the haemoplasma diagnosed cats. Therefore, further studies interested can be conducted to determine the significance of blood load of haemoplasma, retrovirus infection and other associated risk factors affecting the clinical appearances of infection.

ACKNOWLEDGEMENTS

The authors would like to thank their colleagues (Dr. Bilal Akyuz, Dr. Alparslan Yıldırım, Dr. Korhan Arslan, Dr. Nurhan Ertaş, Dr. Şeyda Özkan, Research Asist. E. Gamze Ilgar and Serhat Al) for their help with this study and Dr. Severine Tasker from Bristol University, Department of Small Animal Medicine for gift *M. haemofelis* and '*Candidatus M. haemominutum'* DNAs. Thanks also to Dr. Aytaç Akçay for assistance with the SPSS statistical analysis.

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