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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 out 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 author’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

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 hemoplasma 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).

ÖZET
Bu çalışmanın amacı, kedilerde polimeraz zincir reaksiyonu (PCR) ile haemoplasmozis belirlenmesidir. Çalışmanın materyalini farklı irk ve cinsiyette (41 dişi ve 43 erkek), ortala 5.5 yaşlarında (6 ay-10 yaş) 84 kedinin toplamdan oranları bulunmuştu. Kedi örnekleri hem sitolojik, hem de PCR yöntemi ile inceledi. PCR reaksiyonu için Mycoplasma haemofelis ve Candidatus Mycoplasma haemominutum'un 16S rRNA gen böl-gelerinden sırasıyla 170 bp ve 193 bp bölgesini oluşturuldu. PCR analizi sonrasında 84 kedinin 8’i (%9.52) haemoplasma pozitif olarak belirlendi ve bunlardan 4’ü (%4.76) M. haemofelis, 3’ü (%3.57) Candidatus M. haemominutum ve bir kedı (%1.19) her iki etken açısından pozitif bulundu. Bilgilerimizde bu çalışma ile Türkiye’de kedilerde M. haemofelisin ilk moleküler testi yapılmış ve M. haemofelis ve Candidatus M. haemominutum enfeksiyonlarının birlikte tespiti ilk kez bildirilmiştir.

Anaatır kelimerler: haemoplasmozis, kedi, PCR, sitolojik analiz

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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 emphasis 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 haemominutum 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 anterobrachii with EDTA tubes for thin blood smear, hematological examinations and PCR analysis. All blood samples were stored at -20°C 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 (Novagenetz®, Ankara)) were used producing a 170 bp and a 193 bp amplicon for Mh and CMhm, respectively.

PCR reaction contained 5 µl DNA, 3.5 mM MgCl₂, 2 U Taq polymerase, 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 4º C, followed by 35 cycles of 1 min denaturation at 4º C, 30 sec. primer annealing step at 60º C, and 30 sec. extension at 72º C. In the last cycle, the extension was held at 72º C for 10 min. (15, 16). Thermal cycler (MyGenie® 96 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% agar’s 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 erythrocyte 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-saturated agarose gel (2%) electrophoretogram showing amplified DNA from *M. haemofelis* (170bp) and Candidatus *Mycoplasma haemominutum* (193bp) 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* positive control; 7- *M. haemofelis* positive control, 9- *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).

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

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 leucocyte counts between two groups (*p* > 0.05), but five haemoplasma positive cats had leucocytosis (Table 2).

**DISCUSSION**

Erythrocytic and unculturable haemoplasmas, also known as *Haemobartonella* and *Eperythrozoon*, have the ability to cause seriously haemolytic anemia. Therefore *Haemobartonella* 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 Diff-Quik stain (18,19). The findings of the current study showed that the organisms were seen as purple-blue coccoids and short chains at erythrocyte surface at cyto logical 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...
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Among the cats from a Cat Shelter in Kayseri Metropolitan, these cats may be carriers of haemoplasma infections 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%) were 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 immunosuppression, 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, dyspnea, 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 haematological examinations, they were statistically significant. Besides, these cats may be carrier of haemoplasma infection due to the hemotocrit 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, Izmir) 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

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### Table 2. Haematological values in haemoplasma positive and negative cats ($X_{\leq}S_{\geq}$ cats)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reference Range (17)</th>
<th>Haemoplasma (-)</th>
<th>Haemoplasma (+)</th>
<th>Statistical Significance**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(minimum-maximum)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC (10^9/μL)</td>
<td>5.5-19.5</td>
<td>17.47±1.12</td>
<td>19.49±2.54*</td>
<td>p=0.571</td>
</tr>
<tr>
<td>RBC (10^6/μL)</td>
<td>4.6-10.0</td>
<td>7.95±0.21</td>
<td>12.23±5.00*</td>
<td>p=0.421</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>9.3-15.3</td>
<td>12.56±0.36</td>
<td>11.24±0.34</td>
<td>p=0.012</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>28.0-49.0</td>
<td>38.19±1.02</td>
<td>35.29±0.85</td>
<td>p=0.035</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>39.0-52.0</td>
<td>48.19±0.41</td>
<td>50.21±1.17</td>
<td>p=0.130</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>13.0-21.0</td>
<td>15.74±0.15</td>
<td>15.91±0.29</td>
<td>p=0.705</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>30.0-38.0</td>
<td>32.40±0.32</td>
<td>31.78±0.37</td>
<td>p=0.531</td>
</tr>
<tr>
<td>PLT (10^3/μL)</td>
<td>100-514</td>
<td>227.86±16.04</td>
<td>253.88±49.74</td>
<td>p=0.618</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>2-12</td>
<td>6.70±0.88</td>
<td>5.24±1.46</td>
<td>p=0.599</td>
</tr>
</tbody>
</table>

*:* Different according to the standard value

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


