Prevalence of haemoplasma infection in pet cats from 4 different provinces in Turkey

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SUMMARY

The haemoplasmosis prevalence was investigated in 217 cats (102 healthy and 115 diseased cats with various clinical signs) stemming from 4 provinces of Turkey (Bursa, Izmir, Ankara and Antalya) using cytological examination (Romanowsky stain) and PCR assay. Whereas only 13.36% of cats were positively diagnosed for haemoplasmosis by cytology, the PCR assay has efficiently increased diagnostic gains (positive and negative predictive values were 0.996 and 1.000, respectively). In this way, the prevalence for haemoplasma in the total feline population studied was 18.9%; the diseased animals with clinical signs compatible with haemoplasmosis were significantly more affected (27.8%) than healthy cats (8.8%) and 100% of animals with clinical signs compatible with haemoplasmosis were PCR positive. Besides, the proportion of non anaemic positive cats were relatively elevated (23.9%) and no significant geographical influence was observed. These results demonstrated that PCR assay was a more reliable method than Romanowsky staining for detecting and identifying haemoplasmosis and that this infection appears relatively common in pet cats.

Keywords: Cat, Haemoplasmosis, PCR assay, Cytological analysis, Turkey.

RÉSUMÉ

Prévalence de l’hémoplasmose chez les chats domestiques de 4 provinces distinctes en Turquie

Dans cette étude, la présence d’hémoplasmose a été recherchée chez 217 chats (102 sains et 115 malades présentant des symptômes variés) issus de 4 provinces turques (Bursa, Izmir, Ankara et Antalya) par cytologie (coloration de Romanowsky) et PCR. Alors que la cytologie n’a été positive que dans 13.36 % des cas, la PCR a permis une nette amélioration des gains diagnostiques (les valeurs prédictives positive et négative ont été respectivement de 0.996 et 1.000). Ainsi, la prévalence de l’hémoplasmose au sein de la population féline totale étudiée a été de 18.9 %; les animaux malades ont été significativement plus souvent affectés (27.8 %) que les sains (8.8 %) et 100 % des chats présentant des signes cliniques évocateurs de l’hémoplasmose ont donné un résultat positif par PCR. De plus, la proportion de chats non anémiques et positifs s’est avérée relativement élevée (23.9 %) et aucune influence significative de l’origine géographique des chats n’a été mise en évidence. Enfin, la méthode par PCR n’a révélé que la présence de Candidatus Mycoplasma haemominutum dans les 4 régions. Ces résultats démontrent que la PCR est une méthode plus fiable que la coloration de Romanowsky dans la détection et l’identification des hémoplasmases et que cette infection apparaît relativement fréquente chez les chats domestiques.

Mots clés : Chat, Hémoplasmose, PCR, Analyse cytologique, Turquie.

Introduction

Haemotropic mycoplasmas, also named as haemoplasmas, are the causative agents of infectious anaemia [9] and two different haemoplasma species: Mycoplasma haemofelis (M. haemofelis [11, 14], and “Candidatus Mycoplasma haemominutum” [4, 10, 11, 13] were previously reported in cats. Recently a third specie, “Candidatus Mycoplasma turicensis” [21] has been recognized. The feline haemotropic mycoplasmas (haemoplasmas) were recently reclassified within the genus Mycoplasma and as haemoplasmas [12].

The objective of the present study was to evaluate the prevalence of haemoplasma infection by Polymerase Chain Reaction (PCR) assays capable of differentiating the deoxyribonucleic acid (DNA) of Mycoplasma haemofelis and "Candidatus Mycoplasma haemominutum" from healthy, sick anaemic and non anaemic cats in Ankara, Turkey. A further aim also was to alert clinicians of both academical and private practice origins to the possible occurrence of haemoplasma infection in domestic cats in different provinces of Turkey.

Materials and Methods

ANIMALS

Commercial veterinary clinics and veterinarians in private practice throughout Ankara, Bursa, Antalya and Izmir were recruited to participate to the present study between early January 2007 and late August 2007 and a total of 217 cats (129 males and 88 females), 1 to 14 years old, were included in the present study. Among these cats, the following breeds were represented: Siamese (11), Persian (20), Angora (23), Van (17) and crossed (146). Thirty-nine animals were stemming from Bursa, 121 from Ankara, 40 from Izmir and 17 from Antalya. The epidemiological criterion for selecting cats in the present study was the occurrence of clinical signs compatible with haemoplasmosis (such as anaemia, hyperthermia, anorexia, pale mucous membranes, depression, weakness, icterus and splenomegaly) or the complete absence of any clinical sign (healthy controls).

For all animals, blood samples were collected by venous puncture in tubes containing EDTA and were rapidly send to the Department of Parasitology at the University of Ankara.
Faculty of Veterinary. Thin blood smears were performed by the veterinary practitioners and/or in the laboratory of Parasitology and they were stained by the Romanowsky method [2] for evidencing haemoplasma infection. Samples were considered as positive when 3 or more haemoplasma organisms were detected. In parallel, a complete haemogram (Melet-MS 4 automated cell counter, France, in the Ankara Major City Government Pet Animals Health Center Ankara, Turkey) was determined for all studied cats. Cats were classified as anaemic or non-anaemic according to the haemoglobin concentration (Hb) and the packed cell volume (PCV) was also evaluated. Cats with Hb < 8 g/L and/or with PCV < 29% were considered anaemic. In addition, cats were also classified as healthy or sick on the basis of complete physical examination, historical data and complete blood counts.

DNA EXTRACTION AND PCR ASSAY

For PCR assays, DNA was extracted from whole blood using a DNA extraction kit according to the manufacturer’s instructions (EZ DNA isolation kit, Dr. ZEYDANLI, Ankara, Turkey). As was previously described [6, 7, 15], primers used for PCR reaction targeted the 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 primer, Dr. ZEYDANLI, Ankara, Turkey) and produced a 170 base pair (bp) product from M. haemofelis and a 193 bp amplicon from ‘Candidatus M. haemominutum’. The PCR amplifications were carried out in 25 µL reaction mixtures containing 31 pmol of each primer, 200 µM (Amresco) deoxynucleotide triphosphates (dNTPs), 0.025 units Taq DNA polymerase (Gene Mark), 3.5 mM MgCl2, 1x PZR Buffer (Gene Mark) and 2 µL sample DNA. In PCR apparatus (Biometra T-gradient, Labrepco, United Kingdom), the following reaction conditions were applied: 30 seconds at 94°C, followed by 45 cycles of 30 seconds at 57°C in a first step and of 45 seconds at 72°C in the second step. Initial denaturation for 5 minutes at 94°C before the first cycle and an extension step (10 min at 72°C) after the last cycle were performed. The DNAs from M. haemofelis and ‘Candidatus M. haemominutum’ (gifts from Dr. APPLcyARD from Saskatchewan University, Western College of Veterinary Medicine, Department of Veterinary Pathology, Saskatchewan) were used as positive controls. In addition, a reagent negative control (sterile pure water) was included in each PCR run in an attempt to detect contamination. Thereafter, the reaction products (0.5 µg/mL) were submitted to electrophoresis in 2.5% agarose gel containing ethidium bromide and were visualised by UVP gel documentation system (UVP Inc., Upland, California, USA). The size of the PCR product was compared to the size of known positive control DNAs from M. haemofelis and ‘Candidatus M. haemominutum’ and to a 100 bp DNA ladder (Dr. ZEYDANLI, Ankara, Turkey).

STATISTICAL ANALYSIS

On the basis of PCR assay and haematological status, cats were defined as ‘Candidatus M. haemominutum’ infected healthy cats or ‘Candidatus M. haemominutum’ infected sick cats. Statistical power of the study comprised the evaluation of the infection rates among anaemic and non-anaemic cats and among sick and healthy cats. Results were compared by Pearson Chi Square test with significance defined as p < 0.01.

Results

Among the 217 cats, 102 were healthy (with no clinical history and no anaemia) whereas 115 were considered as diseased animals. The province origins of the diseased cats were as follows; Bursa (n = 16), Ankara (n = 77), Izmir (n = 15) and Antalya (n = 7). As shown in Table I, healthy cats exhibited normal PCV values (usual values: 30 to 46%) and normal haemoglobin concentrations (usual values: 8 to 14 g/L). In the group of diseased cats, 69 were anaemic with low haematocrit and/or low haemoglobin concentration, whereas these 2 parameters were not altered in 46 diseased cats (Table I).

<table>
<thead>
<tr>
<th>Haematological parameters</th>
<th>PCV (%)</th>
<th>Haemoglobin (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 29</td>
<td>30 - 46</td>
<td>&gt; 46</td>
</tr>
<tr>
<td>Healthy cats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bursa (n = 23)</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Ankara (n = 44)</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>Izmir (n = 25)</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Antalya (n = 10)</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Total (n = 102)</td>
<td>0</td>
<td>102</td>
</tr>
<tr>
<td>Diseased cats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bursa (n = 16)</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Ankara (n = 77)</td>
<td>48</td>
<td>29</td>
</tr>
<tr>
<td>Izmir (n = 15)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Antalya (n = 7)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total (n = 115)</td>
<td>69</td>
<td>46</td>
</tr>
</tbody>
</table>

Table I: Number of cats with normal or altered hematocrit values (PCV) and haemoglobin concentrations. The feline population studied (n = 217) is composed by 39 cats from Bursa, 121 from Ankara, 40 from Izmir and 17 from Antalya.
Furthermore, 26 diseased cats (22.6%) exhibited clinical signs (hyperthermia, anorexia, pale mucous membranes, depression, weakness, icterus and splenomegaly) compatible with haemoplasmosis; 19 of them were also anaemic.

The cytological examination of the Romanowsky stained smears demonstrated microscopically evidence of *Haemoplasma* infection in 29 cats (8 healthy and 21 diseased). Moreover, 41 cats (18.9%) (9 healthy and 32 diseased) were haemoplasma positive using PCR assay. All samples from these cats produced a 193 bp DNA fragment characteristic of *Candidatus Mycoplasma haemominutum* whereas the 170 bp DNA fragment specific of *M. haemofelis* was never found. The PCR positive cases included the 29 cases diagnosed by cytology plus 12 other samples, cytologically negative (Table II). Consequently, the relative PCR sensibility and specificity compared to the cytological examination were 100% and 93.6% respectively. The calculated predictive positive and negative values were 0.996 and 1.000 respectively, allowing a great positive diagnostic gain of 0.862 and a moderate negative diagnostic gain of 0.134.

The haemoplasmosis prevalence was significantly higher in diseased cats (27.8%) than in healthy cats (8.8%) \((\chi^2 = 12.73, p < 0.01)\). Among the 69 anaemic cats, 21 (30.4%) exhibited a positive PCR result but this prevalence was not significantly higher than the prevalence observed in non anaemic diseased cats (11/46, i.e. 23.9%). Furthermore, all cats with haemoplasmosis symptoms \((n = 26)\) gave a positive PCR assay (Table III). Although the haemoplasmosis prevalence observed in the Bursa province (7.7%) appeared weak compared to the other prevalences obtained in Izmir (17.5%), Ankara (23.1%) and in Antalya (17.6%) provinces, the origins of the cats exerted no significant effect on haemoplasma infection.

### Discussion

This epidemiological and molecular analysis reports the presence of “*Candidatus Mycoplasma haemominutum*” infections in pet cats from four different locations in Turkey. To the present authors’ knowledge, apart from some limited individual studies, a detailed epidemiological research studying haemoplasmosis in different provinces has not been previously reported in Turkey. Earlier Turkish studies about haemoplasmosis had been solely based on cytological diagnosis \([1, 8, 17]\) before recent developments of molecular diagnostic methods. The first case was recognized in Istanbul in 1993 \([17]\), then other cases were reported in Ankara \([8, 18]\), in Van \([1]\) and in Antalya \([19]\). Based on cytological diagnosis, haemoplasmosis prevalences were

![Table II: Contingency table and relative performance of the PCR assay compared to the cytological examination (Romanowsky staining).](image-url)

![Table III: Number of cats detected positive for haemoplasma by cytological examination and by PCR assay according to their health status and to their geographical origin.](image-url)
20% in Ankara [8] and 14.87% in Van [1]. By contrast, a higher haemoplasma infection rate (30.76%) based on PCR assay conducted on 39 cats, was reported in Ankara by URAL et al. [18]. In the present study, the haemoplasma infection rate obtained in cats from Ankara with the PCR assay (23.1%) was relatively weaker. Nevertheless, like in this previous study, the haemoplasma prevalence determined using PCR assay in the present study (18.89%) was markedly higher than using Romanowsky staining (13.36%). Indeed, this molecular method appeared more efficient than the cytological examination since not only the 29 samples positive with the Romanowsky staining were also positive for PCR, but also 12 other samples were also detected positive with PCR assay. Consequently, the PCR technique has offered diagnostic gains, the marked increase of the positive diagnostic gain strengthening a positive diagnostic of haemoplasmosis and the moderately enhanced negative gain allowing exclusion of this disease. The recent development of PCR analysis provided more reliable and sensitive diagnosis of haemoplasma infection in cats [6, 7, 15]. On the other hand, contrary to cytological examination the identification of haemoplasma species is possible with PCR assay by amplifying specific DNA fragments [21]. In this way, only Candidatus Mycoplasma haemominutum was evidenced in the present study with a prevalence established on the 4 Turkish provinces (Bursa, Izmir, Ankara and Antalya) of 18.89%. These results were comparable to those previously reported in United Kingdom [15] and in USA [6] with prevalences of 18.5% and 19.5% respectively, but they were in disagreement with those obtained in Spain (30%) [3], in Australia (27.9%) [16] and in Canada (38%) [7] using also PCR assay. In most of these studies, similar PCR assays and similar primer design were used. Consequently, the differences of haemoplasma infection prevalence suggest the occurrence of geographical influences [15]. Nevertheless, the blood sampling modalities, the nature of the feline population studied (proportions of anaemic cats or of cats with clinical signs compatible with haemoplasmosis) and the PCR conditions (i.e. primers and PCR cycles) may affect PCR results and interfere with the determination of the prevalence [7, 15]. In the present study, the prevalence was significantly higher in diseased cats (27.83%) than in healthy cats (8.82%). A positive PCR result was obtained in 100% of cats with haemoplasmosis clinical signs whereas only 6.7% of diseased cats without haemoplasmosis symptoms (6/89) were positively diagnosed with PCR assay; besides, although the differences were not statistically significant, the frequency of haemoplasma infection appeared greater in anaemic cats (30.4%) than in non anaemic animals (23.9%).

Except CRIADO et al. [3] in Spain and WATANABE et al. [20] in Japan in where high prevalences of infection with M. haemofelis were reported (66% and 57%), the proportions of cats infected by Candidatus M. haemominutum were usually higher than those of animals infected by M. haemofelis [5, 6, 15]. In the present study, no infection with M. haemofelis was revealed by the PCR assay. Consequently, a large proportion of the feline population was exposed to Candidatus M. haemominutum as it was previously described [15]. In addition, cats may have been in a carrier stage following ‘Candidatus M. haemominutum’ infection which then may result in elimination of M. haemofelis and PCR negative status against M. haemofelis [15]. Moreover, the Candidatus M. haemominutum infection has also occurred in healthy cats with a low prevalence, suggesting that this haemoplasma infection is relatively common in pet cats in Turkey and would be underestimated in clinical activities. In addition, diseased animals were significantly more often infected than healthy cats. It would be possible that a depressed health status may promote haemoplasma infection through depletion of defence systems of the organism. The higher risk of diseased cats to be infected with ‘Candidatus M. haemominutum’ may also be explained with precedent exposition of cats to ‘Candidatus M. haemominutum’.

As a conclusion, the PCR assay allows a more efficient detection of haemoplasma in cats than cytological examination and can distinguish the haemoplasma species contrary to the Romanowsky stain. Haemoplasmosis appears to be a relative common infection in pet cats in Turkey affecting healthy and mainly diseased cats.

### Acknowledgments

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