Diagnosis of feline haemoplasma infection using a real-time PCR assay

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ABSTRACT
Haemobartonella felis has been reclassified within the genus Mycoplasma as Mycoplasma haemofelis and ‘Candidatus Mycoplasma haemominutum’, collectively referred to as the feline haemoplasmas. A total of 78 cats from the Johannesburg area that had blood samples submitted to a private veterinary laboratory were tested using a real-time polymerase chain reaction (PCR) assay able to detect and distinguish the two feline haemoplasma (basonym Haemobartonella) species. All samples had been diagnosed with haemoplasma infection by cytological examination of blood smears. Statistical analysis was performed to evaluate associations between haemoplasma status, age, and haematological and biochemical parameters. On PCR assay 43 cats (55 %) were haemoplasma negative, 25 (32.1 %) positive for ‘Candidatus Mycoplasma haemominutum’, 5 (6.4 %) positive for Mycoplasma haemofelis and 5 (6.4 %) positive for both species. Significant inverse correlation was found between the amount of M. haemofelis DNA present in the blood and the haematocrit value. Cats that were positive for M. haemofelis showed macrocytic regenerative anaemia, monocytosis and thrombocytopenia. This report documents the existence of both haemoplasma species in cats in South Africa.

Key words: cat, haematology, Haemobartonella felis, haemoplasma, PCR.


INTRODUCTION
Based on phylogenetic analysis of 16S rRNA gene sequences, Haemobartonella felis has been reclassified within the genus Mycoplasma as Mycoplasma haemofelis 1 and ‘Candidatus Mycoplasma haemominutum’ 11. Both species are collectively referred to as the feline haemoplasmas. Experimental infection with M. haemofelis often causes a severe haemolytic anaemia 2,7,22. Conversely, ‘Candidatus M. haemominutum’ infection does not usually induce anaemia 2,7,22, although it has been suggested that co-infection with ‘Candidatus M. haemominutum’ and feline retroviruses may result in anaemia 2.

The existence of feline haemoplasmas in South African cats was first reported in 1942 2. No further studies have, however, been reported in the literature. Until recently, cytological examination of blood smears to visualise organisms on the surface of erythrocytes was relied upon to diagnose feline haemoplasma infection. Based on cytological diagnosis, feline haemoplasma infection has been recognised worldwide 2,7,11,22,24.

Recently, several studies have shown that conventional polymerase chain reaction (PCR) assays are more sensitive than cytology for the diagnosis of this infection 2,7,22. In addition, PCR, unlike cytology, is able to definitively distinguish between M. haemofelis and ‘Candidatus M. haemominutum’ infection. Recent prevalence studies using conventional PCR to detect feline haemoplasma infection have been carried out in the USA 11, the United Kingdom 21, and Australia 22.

Real-time PCR allows the detection of amplicon accumulation as it is synthesised, so post-amplification steps, such as gel electrophoresis, is not required. The assay is therefore performed rapidly, and, since there is no need to open the reaction tubes following PCR, amplicon carry-over and false-positive results are far less likely than with conventional PCR. The amount of fluorescence generated in real-time PCR is proportional to the amount of accumulated PCR product, so measurement of fluorescence during the exponential phase of PCR provides an accurate means to quantify DNA template in an unknown sample. Quantification of DNA template may be of particu-lar importance with infectious agents such as the feline haemoplasmas, which do not invariably cause clinical disease in the host. A real-time PCR assay for the detection and quantification of feline haemoplasma DNA has recently been described 21.

The aim of this study was to evaluate the prevalence of infection with both M. haemofelis and ‘Candidatus M. haemominutum’ in blood samples collected from cats in the Johannesburg area, using a real-time PCR assay, and to correlate the results with haematological and serum biochemical parameters.

MATERIALS AND METHODS

Samples
Surplus EDTA-anticoagulated blood (minimum 100 µl) from 78 feline samples submitted to a private veterinary laboratory (Golden Veterinary Laboratories, Alberton, South Africa) for routine haematological testing was stored at −20 °C. The samples were obtained from cats with a variety of disease conditions and all had been diagnosed with haemoplasmosis on blood smear evaluation, stained with Cams Quick stain (CA Milsch, Krugersdorp, South Africa). The batched blood samples were then couriered to the Department of Veterinary Clinical Science, University of Bristol in the United Kingdom.

For each sample, the cat’s age, haematological data, and any available biochemical data were recorded.

DNA extraction
Genomic DNA was prepared from 100 µl of the EDTA whole blood samples using the DNeasy 96 Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions. For each plate of DNA extractions performed, three 100 µl aliquots of phosphate buffered saline underwent the DNA extraction protocol for subsequent PCR to screen for contamination during DNA extraction.

PCR amplification
All PCR amplifications were performed in 25 µl reaction volumes. A feline haemoplasma real-time PCR assay 28 was performed on all samples. Briefly, this assay comprised feline haemoplasma-specific primers (Hf Forward 5'-ACGAAGTCT GATGGAGCAATA-3' and Hf Reverse 5'-ACGCCATATAATCGRATAAT-3')
phosphatase (ALP), urea, and creatinine) and 28S rDNA real-time threshold cycle (C\textsubscript{T}) values. Based on the results of the real-time haemoplasma PCR assay, cats were divided into four groups: negative, ’Candidatus M. haemominutum’-only positive, M. haemofelis-only positive, and dual positive (co-infected cats). These four groups were statistically analysed for differences in age, 28S rDNA C\textsubscript{T} values, haematological or biochemical variables.

Normally distributed data were analysed using analysis of variance (ANOVA) and non-normally distributed data were analysed using Kruskal-Wallis ANOVA. Statistical evaluation was carried out using SPSS for Windows 10.1.0 (SPSS Inc., Chicago, Illinois). Descriptive statistics were obtained for each variable and normality was tested for using the Kolmogorov-Smirnov test. Logarithmic transformation of some variables with a skewed distribution resulted in normal distributions for parametric analysis. These variables were WCC, band neutrophil, reticulocyte and platelet counts, ALT, SAP, urea, and creatinine concentrations. Square root transformation of the eosinophil counts resulted in a normal distribution. Logarithmic and square root transformation of both lymphocyte and basophil counts did not result in normal distributions, necessitating non-parametric analysis. Although normally distributed data and the Mann-Whitney U-test for non-normally distributed data. A significant association was taken to be one with a P value ≤ 0.05. Bivariate correlation between haemoplasma DNA and Ht was measured by determination of Spearman’s correlation coefficients.

RESULTS

Haemoplasma real-time PCR

Seventy-eight blood samples underwent real-time PCR. Twenty-five cats (32.1 %) were PCR positive for ’Candidatus M. haemominutum’ alone, 5 (6.4 %) were positive for M. haemofelis alone, and 5 (6.4 %) were dual positive (co-infected) for both species (Fig. 1). Overall, of the 78 samples, 35 (44.9 %) were positive for one or both feline haemoplasma species. All positive and negative controls in the PCR assays were positive and negative, respectively.

Feline 28S rDNA real-time PCR

All samples submitted for haemoplasma real-time PCR gave positive results with the feline 28S rDNA assay, indicating the presence of amplifiable DNA. The C\textsubscript{T} values ranged from 22.4 to 41.3 (mean 27.4, median 26.6, standard deviation 3.6, standard error 0.41). The sample with a C\textsubscript{T} value of 41.3 generated a positive result with the haemoplasma real-time PCR assay for ’Candidatus M. haemominutum’. All water controls gave negative results. No significant differences in 28S rDNA C\textsubscript{T} values were found between the four groups based on haemoplasma PCR results.

Characteristics of cases

Descriptive statistics for continuous
variables, including age, haematological and biochemical parameters are shown in Table 1.

**Age**

No significant difference in age was found between the four groups based on haemoplasma PCR results (Fig. 2).

**Haematological parameters**

Of the haematological variables evaluated by ANOVA or Kruskal-Wallis ANOVA, six were significantly different between the four groups based on haemoplasma PCR results. These were RBC ($P < 0.001$), Hb ($P = 0.001$), Ht ($P = 0.001$), monocyte count ($P = 0.04$) and platelet count ($P = 0.02$) (Fig. 3).

The RBC counts of the *M. haemofelis*-only group were significantly lower than both the *Candidatus M. haemominutum*-only group ($P = 0.001$) and the negative group ($P < 0.001$) while the dual-positive group RBC counts were significantly lower than those of the negative group only ($P = 0.02$). There was no significant difference in the RBC counts of the negative group and the *Candidatus M. haemominutum*-only group ($P = 0.36$).

The Hb values of the *M. haemofelis*-only group were significantly lower than those of all the other groups; the dual-positive group ($P = 0.009$), the *Candidatus M. haemominutum*-only group ($P = 0.002$) and the negative group ($P < 0.001$). The Hb values of the dual-positive group were also significantly lower than those of the negative group ($P = 0.02$) but significance was not reached with the *Candidatus M. haemominutum*-only group ($P = 0.09$).

There was no significant difference in the Ht values of the negative group and the *Candidatus M. haemominutum*-only group ($P = 0.44$).

Bivariate analysis revealed a significant correlation between the Ht and DNA from *M. haemofelis*-positive cats (Spearman $r = 0.82$, $P = 0.023$) but no significant correlation was found between the Ht and DNA from *Candidatus M. haemominutum*-positive cats.

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**Table 1: Descriptive statistics for haematological and serum biochemistry variables in cats with natural haemoplasma infection.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Median</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64</td>
<td>1.0</td>
<td>19.0</td>
<td>6.0</td>
<td>7.5</td>
<td>5.11</td>
<td>0.64</td>
</tr>
<tr>
<td>Haematocrit (l/l)</td>
<td>78</td>
<td>8.0</td>
<td>47.0</td>
<td>29.0</td>
<td>27.75</td>
<td>8.77</td>
<td>0.99</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>78</td>
<td>26</td>
<td>163</td>
<td>105</td>
<td>97</td>
<td>32</td>
<td>3.6</td>
</tr>
<tr>
<td>Red cell count ($x 10^{12}$/l)</td>
<td>78</td>
<td>1.44</td>
<td>11.70</td>
<td>6.00</td>
<td>6.50</td>
<td>2.39</td>
<td>0.27</td>
</tr>
<tr>
<td>Mean cell volume (fl)</td>
<td>78</td>
<td>30</td>
<td>72</td>
<td>43</td>
<td>44.5</td>
<td>7.39</td>
<td>0.64</td>
</tr>
<tr>
<td>Mean cell haemoglobin concentration (g/dl)</td>
<td>78</td>
<td>0.13</td>
<td>48.30</td>
<td>7.86</td>
<td>6.31</td>
<td>2.75</td>
<td>1.12</td>
</tr>
<tr>
<td>White cell count ($x 10^{9}$/l)</td>
<td>78</td>
<td>9</td>
<td>844</td>
<td>155.5</td>
<td>118.0</td>
<td>3.09</td>
<td>1.14</td>
</tr>
<tr>
<td>Platelets ($x 10^{9}$/l)</td>
<td>70</td>
<td>0.4</td>
<td>20.7</td>
<td>5.13</td>
<td>6.25</td>
<td>4.38</td>
<td>0.52</td>
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<td>Neutrophils ($x 10^{9}$/l)</td>
<td>70</td>
<td>1.89</td>
<td>0.33</td>
<td>0.42</td>
<td>0.34</td>
<td>0.04</td>
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<tr>
<td>Lymphocytes ($x 10^{9}$/l)</td>
<td>70</td>
<td>44</td>
<td>0.72</td>
<td>1.80</td>
<td>5.32</td>
<td>0.63</td>
<td>0.63</td>
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<tr>
<td>Monocytes ($x 10^{9}$/l)</td>
<td>70</td>
<td>0</td>
<td>0.39</td>
<td>0.47</td>
<td>0.35</td>
<td>0.04</td>
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<tr>
<td>Eosinophils ($x 10^{9}$/l)</td>
<td>70</td>
<td>3.68</td>
<td>0.11</td>
<td>1.06</td>
<td>0.18</td>
<td>0.03</td>
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<tr>
<td>Basophils ($x 10^{9}$/l)</td>
<td>70</td>
<td>0.28</td>
<td>0</td>
<td>0.04</td>
<td>0.06</td>
<td>0.01</td>
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<tr>
<td>Reticulocytes %</td>
<td>75</td>
<td>0.20</td>
<td>2.0</td>
<td>1.95</td>
<td>1.04</td>
<td>0.09</td>
<td>0.09</td>
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<tr>
<td>Total serum protein</td>
<td>62</td>
<td>45</td>
<td>98</td>
<td>70.44</td>
<td>10.48</td>
<td>6.15</td>
<td>0.79</td>
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<tr>
<td>Albumin</td>
<td>61</td>
<td>12</td>
<td>47</td>
<td>27.44</td>
<td>6.15</td>
<td>9.96</td>
<td>1.28</td>
</tr>
<tr>
<td>Globulin</td>
<td>61</td>
<td>29</td>
<td>74</td>
<td>40.15</td>
<td>9.96</td>
<td>1.28</td>
<td>1.28</td>
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<tr>
<td>ALT</td>
<td>59</td>
<td>5</td>
<td>1033</td>
<td>74.87</td>
<td>2.77</td>
<td>1.14</td>
<td>1.14</td>
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<tr>
<td>ALP</td>
<td>54</td>
<td>10</td>
<td>317</td>
<td>35.49</td>
<td>2.27</td>
<td>1.12</td>
<td>1.12</td>
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<tr>
<td>Urea</td>
<td>58</td>
<td>1.9</td>
<td>61.5</td>
<td>8.93</td>
<td>2.07</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>Creatinine</td>
<td>59</td>
<td>32</td>
<td>949</td>
<td>131.1</td>
<td>2.03</td>
<td>1.10</td>
<td>1.10</td>
</tr>
</tbody>
</table>

*aGeometric mean, standard deviation (SD) and standard error (SE).

*bSquare root transformed mean, standard deviation (SD) and standard error (SE).

*cVariables with non-normally distributed data.

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Fig. 2: Age distribution of 78 cats with natural haemoplasma infection grouped by haemoplasma PCR result. CMhm = *Candidatus M. haemominutum*; Mhf: *Mycoplasma haemofelis*. Boxes represent the 25th, 50th (median) and 75th quartiles with whiskers extending to the greatest and smallest values.
correlation was evident between the Ht and DNA from cats positive for ‘Candidatus M. haemominutum’ (Spearman $r = 0.14$, $P = 0.448$).

The MCV values of both the M. haemofelis-only and the dual-positive groups were significantly greater than those of the negative group ($P = 0.005$ and $P = 0.01$, respectively). No other significant differences in MCV were found between the four groups.

The monocyte count of the M. haemofelis-only group was significantly greater ($P = 0.03$) than that of the negative group. No other significant differences in monocyte counts were found between the four groups.

The M. haemofelis-only group had lower platelet counts than the negative group ($P = 0.05$). No other significant differences were found between the groups.

Fig. 3: Distribution of selected haematological variables in cats with natural haemoplasma infection grouped by haemoplasma PCR result. CMhm = ‘Candidatus M. haemominutum’; Mhf = Mycoplasma haemofelis. Boxes represent the 25th, 50th (median) and 75th quartiles with whiskers extending to the greatest and smallest values. Black dots indicate outliers (cases with values greater than 1.5 box lengths from the upper or lower edge of the box).
Serum biochemical parameters

None of the biochemical variables evaluated showed any significant differences between the four groups based on haemoplasma PCR results.

DISCUSSION

This is the first study in South African cats to report the prevalence of both 'Candidatus M. haemominutum' and M. haemofelis in a convenience-sample of feline patients. The successful amplification of haemoplasma DNA in these specimens confirms, for the first time, the existence of both 'Candidatus M. haemominutum' and M. haemofelis in South Africa. Indeed, sequencing of the full length of the 16S rRNA gene of a number of South African haemoplasma isolates has confirmed the existence of species with near identical 16S rRNA sequences to those previously reported in the UK, USA and Australia.

There is currently no recognised accepted gold standard definitive diagnostic method for the diagnosis of haemoplasma infections. PCR has been shown to be more sensitive than cytology for diagnosis in several studies with positive PCR results obtained with samples negative on cytology, including experimentally infected cats. To the best of our knowledge the primers used in the assays of the current studies are known to be specific for haemoplasma species. They do not amplify template DNA from other bacterial organisms associated with bacteraemia in cats, and sequence data from different worldwide isolates show that these primers are specific to regions of the 16S rRNA gene, which are conserved in all the isolates examined. The fluorogenic probes used in the real-time PCR assay enable distinction between M. haemofelis and 'Candidatus M. haemominutum'. Although morphological differences between the species have been described, examination of blood smears cannot reliably distinguish between the species. Additionally, cytology is based on subjective interpretation of erythrocyte-associated bodies. False positive cytology diagnoses have been reported when feline erythrocitic inclusions such as Howell-Jolly bodies or stain precipitate are mistaken for haemoplasma organisms.

The present study was carried out using a convenience-sampled population, the limitations of which have been discussed previously. When assessing prevalence of infection, truly random samples should be used to generate data, but such samples are difficult to obtain in companion animal studies. Without doubt, differences exist in the sex, age and breed distributions of the cats sampled compared with the general South African cat population. The cats studied also differ from cats in the general population because the majority were being investigated by veterinarians.

In previous studies the prevalence of 'Candidatus M. haemominutum' infection was 23.1 % in Australia, 12.7 % in the USA and 16.9 % in the UK. M. haemofelis infection was less common (4.1 % in Australia, 4.5 % in the USA and 1.4 % in the UK) and dual infection was rare (0.7 % of cats in Australia, 2.3 % in the USA and 0.2 % in the UK). In this study 'Candidatus M. haemominutum' infection was 32.1 %, M. haemofelis infection was 6.4 %, and dual infection was 6.4 %. Direct comparisons of the prevalence reported in these different studies are of limited value because of likely differences in the populations sampled. Indeed, the USA and Australian studies as well as the current study were performed on samples received after requests for blood samples from suspected haemoplasma-infected cats were made to local veterinarians. In the USA study, 37 % of cats evaluated for haemoplasma infection by PCR were suspected of harbouring haemoplasmas based on the presence of anaemia, fever and/or cytological evidence of infection. This study included all cats that had been diagnosed with feline haemoplasma infection based on cytology. Limitations therefore exist with extrapolation of results from this study to the general cat population in South Africa.

The clinical significance of a positive PCR result for 'Candidatus M. haemominutum' is not known. Studies have shown a relatively high prevalence in cats, both in healthy and sick groups, confirming that infection with this organism is quite common in different parts of the world. A positive PCR result for 'Candidatus M. haemominutum' does not indicate that there is disease associated with infection. In studies in experimentally infected cats, mild or no pathogenicity associated with this species has been documented, and in naturally infected cats there was no significant difference in prevalence of infection with 'Candidatus M. haemominutum' between anaemic and non-anaemic cats. None of the haematological variables measured in either this study or in the UK study showed significant statistical differences between 'Candidatus M. haemominutum'-infected cats and negative cats.

In the present study, both M. haemofelis-infected cats and those co-infected with both haemoplasma species had significantly lower RBC counts, Hb and Ht values, and significantly higher MCV values, compared with negative cats. Thus, an association between M. haemofelis and a regenerative anaemic status could be hypothesised from these results. It has been suggested in one experimental study that dual infection with both haemoplasma species may result in more severe disease than infection with M. haemofelis only, since co-infected cats showed more pronounced clinical abnormalities in terms of severity and duration of anaemia, variations in body temperature and leucocyte abnormalities although they did not show more severe haematological changes than those infected with M. haemofelis alone. Indeed, the M. haemofelis-alone-infected group had significantly lower Hb and Ht values than the co-infected group. No significant association between M. haemofelis and anaemia was found in the UK study whereas an association was found in the Australian study. Such differences could simply be a reflection of sample numbers in the studies or varying pathogenicity between different M. haemofelis isolates from different parts of the world. Since some healthy, non-anaemic cats were found to be M. haemofelis infected in the present study, a positive PCR result for M. haemofelis, as for 'Candidatus M. haemominutum', does not indicate that there is disease associated with infection.

The relatively low number of cats infected with M. haemofelis in these studies limits the reliable determination of associations with infection. In the UK study, there was no significant difference in age between the M. haemofelis-infected and the negative groups, although a significant difference was seen between the M. haemofelis and the 'Candidatus M. haemominutum'-infected cats, due to the latter being older. In the UK study, a significant association between FIV status and M. haemofelis infection was found, but as described for 'Candidatus M. haemominutum', other factors such as breed may be confounding effects. Further studies using larger numbers of M. haemofelis-infected cats will be required to characterise true associations.

Positive PCR results (for both haemoplasma species) have been reported in asymptomatic cats, therefore the significance of a positive PCR result should always be interpreted in the light of observed physical findings and haematological features of the patient. Real-time PCR assays offer quantitative information on the amount of haemoplasma DNA present in addition to confirming the presence of haemoplasma DNA. In this study it was hypothesised that those cases in which haemoplasma infection was thought to be the cause of the anaemia
should have the greatest quantities of haemoplasma DNA present in blood. The correlation data enabled evaluation of the relationship between Ht values and the quantity of haemoplasma DNA present in blood samples. Significant positive correlation was seen with M. haemofelis-infected cats but not with the 'Candidatus M. haemominutum'-infected cats. This is not surprising given the differences in pathogenicity reported for the two species. The significant positive correlation reported in the M. haemofelis-infected cats occurred despite the inclusion of only a very small number of cats.

Future studies should further evaluate the use of quantified haemoplasma DNA data from a larger and more representative cohort of cats since this may help with the interpretation of the significance of a positive PCR result, particularly with M. haemofelis. In particular, quantified data may help distinguish between chronic asymptomatic haemoplasma carrier cats and those with active infection.

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