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3 The Prevalence of Three Species of Feline Haemoplasmas in Samples  
4 Submitted to a Diagnostics Service as Determined by Three Novel Real-  
5 Time Duplex PCR Assays

6

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## 1 Abstract

2 Three distinct species of feline haemoplasmas are recognised; *Mycoplasma*  
3 *haemofelis* (Mhf), ‘*Candidatus Mycoplasma haemominutum*’ (CMhm) and  
4 ‘*Candidatus Mycoplasma turicensis*’ (CMt). These species differ in pathogenicity as  
5 Mhf and CMt can be associated with anaemia whereas CMhm usually results in few  
6 clinical signs. The purpose of this study was to develop quantitative real-time PCR  
7 assays for the detection of all three feline haemoplasma species combined with an  
8 endogenous internal control and to determine the prevalence of infection, using these  
9 assays, in 1592 EDTA blood samples submitted to Langford Veterinary Diagnostics,  
10 University of Bristol for haemoplasma testing.

11 Primers and TaqMan probes were designed against published 16S rDNA  
12 sequences. These assays were combined with a feline 28S rDNA-specific assay to  
13 produce three duplex assays. The assays detected 1-10 copies of a sequence-specific  
14 plasmid per PCR. None of the assays showed cross-reactivity with  $10^6$  copies of a  
15 sequence-specific plasmid from the non-target haemoplasma species.

16 Real-time PCR was performed on all samples using the three assays. Seven  
17 samples were negative for feline 28S rDNA and were excluded from the study. Of  
18 the remaining 1585 samples, 45 (2.8%), 177 (11.2%) and 27 (1.7%) samples were  
19 positive for Mhf, CMhm and CMt respectively, including 11 Mhf/CMhm, 10  
20 CMhm/CMt and two Mhf/CMt dual infections and two triple infections. The results  
21 of this study demonstrate the utility of these new duplex PCR assays for the detection  
22 of haemoplasma infections. CMhm was the most common infection and CMt  
23 infections were often associated with co-infection with other haemoplasma species,  
24 especially CMhm.

## 1 Introduction

2 Feline infectious anaemia is caused by feline haemotropic mycoplasmas, also  
3 known as haemoplasmas. Three distinct species of feline haemoplasmas are  
4 recognised; *Mycoplasma haemofelis* (Mhf), ‘*Candidatus Mycoplasma*  
5 *haemominutum*’ (CMhm) and ‘*Candidatus Mycoplasma turicensis*’ (CMT) (Foley and  
6 Pedersen, 2001; Neimark et al., 2001, 2002; Willi et al., 2005). These three species  
7 differ in their pathogenicity with Mhf inducing the most severe anaemia (Foley et al.,  
8 1998, Willi et al., 2005). All three species have been documented in cats in recent  
9 epidemiological studies from a number of countries (Lobetti and Tasker, 2004; Tasker  
10 et al., 2003a; Tasker et al., 2004; Willi et al., 2006a; Willi et al., 2006b).

11 The inability to culture feline haemoplasmas *in vitro* has severely limited the  
12 development of protein-based diagnostic assays and has led to the use of molecular  
13 methods of diagnosis such as PCR assays. Recently, quantitative real-time PCR  
14 (qPCR) assays have been described for the diagnosis of all three feline haemoplasma  
15 species (Sykes et al., 2007; Tasker et al., 2003b; Willi et al., 2006a; Willi et al., 2005).  
16 A limitation of these qPCR assays is that they lack an endogenous internal control  
17 multiplexed assay to confirm the presence of amplifiable sample DNA and the  
18 absence of PCR inhibitors. Additionally these qPCR assays do not use specific  
19 primers for each of the three haemoplasma species (Sykes et al., 2007; Tasker et al.,  
20 2003b; Willi et al., 2006a; Willi et al., 2005) which is preferred to ensure accurate  
21 detection and quantification of infection in samples containing more than one  
22 haemoplasma species. The aim of the current study was to develop species-specific  
23 TaqMan probe-based qPCR assays that could be multiplexed with a feline 28S rDNA  
24 assay previously developed in our laboratory (Helps et al., 2005) for the diagnosis of  
25 feline haemoplasma infection. In addition, these new assays were to be applied to

- 1 feline blood samples submitted to Langford Veterinary Diagnostics, University of
- 2 Bristol for haemoplasma testing between April 2002 and July 2006 in order to
- 3 determine the prevalence of each haemoplasma species in these samples.

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## 1 **Materials and Methods**

### 2 **Primer and Probe Design**

3 A consensus 16S rDNA sequence for each of the three feline haemoplasma  
4 species (Mhf, CMhm and CMT) was produced by aligning all available GenBank  
5 sequences using DS Gene 1.5 (Accelrys, Cambridge, UK). Feline haemoplasma  
6 species-specific primers and probes were designed with Primer 3 (Rozen and  
7 Skaletzky 2000) ([www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).) using  
8 the three consensus sequences. The primer and probe sets were designed such that the  
9 annealing temperatures of the primers were 60°C and the probes 8-10°C higher with  
10 an 80 to 150bp product (Table 1). In order to minimise primer-dimer formation, the  
11 maximum self-complementarity score was set at 4 and the maximum 3' self-  
12 complementarity score was set at 2. Each of the three haemoplasma assays was  
13 combined with a previously validated feline 28S rDNA-specific assay to produce  
14 three duplex assays (Helps et al., 2005).

### 15 **Samples and DNA Extraction**

16 DNA was extracted from 1592 blood samples submitted to Langford  
17 Veterinary Diagnostics, University of Bristol for feline haemoplasma testing between  
18 April 2002 and July 2006. The samples were anonymised prior to analysis so the  
19 results of previous PCR analysis, haematology, blood biochemistry or clinical history  
20 were not available. DNA was extracted from 100µl EDTA blood using either the  
21 DNeasy Blood and Tissue Kit (Qiagen Ltd., Crawley, UK) or the Macherey-Nagel  
22 Nucleospin Blood kit (ABgene, Epsom, UK) as per the manufacturer's protocol. The  
23 DNA was eluted with 100µl of elution buffer.

## 1       **Real-time PCR (qPCR)**

2           Real-time PCR was performed using Qiagen HotStarTaq Master Mix  
3 (Crawley, UK) with 200 nM of each primer, 100 nM of each probe or SYBR Green I  
4 (1:100,000 final dilution) (Sigma-Aldrich Ltd., Poole, Dorset), 4.5 mM MgCl<sub>2</sub> and 5  
5 µl of DNA in a total volume of 25µl. All reactants were mixed together as a master  
6 mix and aliquotted into 24 or 96-well PCR plates (Thermofast, Abgene, Epsom,  
7 Surrey) prior to addition of the 5µl sample. Positive control samples of known copy  
8 number and a negative control (water) were included in each run.

9           The PCR was performed in an iCycler IQ (Bio-Rad Laboratories Ltd, Hemel  
10 Hempstead, UK) with an initial incubation of 95°C for 15 min and then 45 cycles of  
11 95°C for 10s and 60°C for 30s during which the fluorescence data were collected.  
12 The thermocycling protocol was extended when SYBR Green I was used by heating  
13 the samples from 75°C to 95°C in 0.5°C increments with a dwell time at each  
14 temperature of 15 seconds during which time the fluorescent data were collected in  
15 order to create a melting curve. The melting temperatures of the products was  
16 determined with the iCycler iQ Optical System Software (version 3: Bio-Rad  
17 Laboratories Ltd.) using a rate of change of fluorescence (-d(RFU)/dT) versus  
18 temperature graph.

19           Previously sequenced plasmids containing the entire 16S rDNA gene from  
20 each of the target species (Tasker et al., 2003c; Willi et al., 2005) were used to test the  
21 efficiency and specificity of the reactions. A ten-fold serial dilution of each plasmid  
22 (range: 1 to 10<sup>7</sup> copies/reaction), mixed with an equal volume of genomic DNA  
23 extracted from 200µl of EDTA blood from a specific pathogen free cat, was used to  
24 calculate the reaction efficiency for each of the haeomplasma-specific assays. A

1 master mix was made up for the qPCR reactions before they were aliquotted in  
2 triplicate into the PCR plate prior to addition of the template into each reaction tube  
3 individually. A graph of threshold cycle (Ct) versus  $\log_{10}$  relative copy number of the  
4 sample from the dilution series was produced and the slope of this graph was used to  
5 determine the reaction efficiency. These reaction efficiencies were used to calculate  
6 the number of gene copies present in the assayed samples relative to a plasmid  
7 dilution of known copy number ( $10^3$  16S rDNA copies per PCR) which was included  
8 in each sample run. The number of gene copies in the unknown samples was  
9 calculated using the  $E^{\Delta C_t}$  equation. The Ct results of the plasmid samples were  
10 comparable to those obtained with the standard curve experiment to calculate the  
11 reaction efficiency.

12 During the optimisation of the assays, reaction products from the sequence-  
13 specific plasmids and infected blood samples were separated using 2% agarose gel  
14 (Molecular Biology Grade Agarose, Helena Biosciences, Sunderland, UK)  
15 electrophoresis to confirm the size and number of amplicons produced.

## 16 **Statistical Analysis**

17 The 95% confidence intervals (CI) of the observed prevalences and statistical  
18 comparison of prevalences (2 sample *t*-test) between studies were calculated using  
19 Minitab version 13 (Minitab Inc., Coventry, U.K.).

## 1 Results

### 2 Assay Optimisation

3 The haemoplasma species-specific assays were designed with mismatches  
4 with the non-target species in both the primer and probe sequences (Table 2). The  
5 mismatches in the primer sequences were concentrated towards the 3' end of the  
6 sequence. The specificity of the assays was tested by adding  $10^6$  copies of plasmids  
7 containing 16S rDNA sequences from each of the three species. No cross-reactivity  
8 was seen between the assays and the plasmids of the non-target species with both the  
9 assay's TaqMan probe and SYBR Green I, indicating that the primer sets alone were  
10 species-specific. The assays produced a single melt peak when run with SYBR Green  
11 I and a single amplicon of expected size was produced when separated by agarose gel  
12 electrophoresis (data not shown).

13 The reaction efficiencies of the assays were assessed in the duplex format  
14 using serial ten-fold dilutions of the target species plasmid over 8 orders of  
15 magnitude. These plasmids were diluted in a background of feline DNA in order to  
16 give a positive result with both assays in the duplex with a 28S rDNA result similar to  
17 those measured in the clinical samples. All reactions had efficiencies in excess of  
18 91% (Table 1) and they were able to detect between 1 and 10 plasmid copies per PCR.

### 19 Clinical Samples

20 The number of haemoplasma PCR-positive samples and the 16S rDNA copy  
21 numbers for each haemoplasma species are shown in Table 3. All of the 222  
22 haemoplasma positive samples were also positive for feline 28S rDNA, although the  
23 Ct value was increased in some of the samples with CMhm or Mhf (but not CMt) 16S

1 rDNA copy number in excess of  $10^6$ . This inhibition of the feline 28S rDNA assay  
2 was proportional to the amount of CMhm or Mhf 16S rDNA present above  $10^6$   
3 copies. The feline 28S rDNA assay was not inhibited when the samples were run  
4 with the assays for the haemoplasma species not present as the Ct values were  
5 comparable to other samples. Similar feline 28S rDNA results were obtained from  
6 samples processed with the DNeasy and Nucleospin extraction kits.

7         Seven of the 1592 samples analysed tested only weakly positive or were  
8 negative for feline 28S rDNA (4 samples with  $Ct > 35$ , 3 negative samples) and were  
9 negative for all three haemoplasma species. These seven samples were excluded from  
10 the study as the negative haemoplasma results may have been due to the absence of  
11 amplifiable DNA or the presence of PCR inhibitors (some of these samples had slight  
12 haemoglobin discolouration). Of the 1585 samples remaining in the study, 45 (2.8%),  
13 177 (11.2%) and 27 (1.7%) were positive for Mhf, CMhm and CMt, respectively  
14 (Table 3).

15         A number of recent studies have reported the prevalence of the three  
16 haemoplasma species in feline blood samples from the UK, Switzerland, Australia  
17 and South Africa (Lobetti and Tasker, 2004; Tasker et al., 2003a; Tasker et al., 2004;  
18 Willi et al., 2006a; Willi et al., 2006b). The prevalences reported in these studies and  
19 the calculated 95% CI are detailed in Table 4. The prevalence of Mhf infection found  
20 in the present study was not significantly different from those reported in samples  
21 from the UK, Switzerland or Australia ( $p > 0.05$ ) but was significantly lower than in  
22 samples from South Africa ( $p < 0.001$ ). The prevalence of CMhm infection found in  
23 the present study was not significantly different from that reported in samples from  
24 Switzerland but was significantly lower than that reported in samples from the UK  
25 ( $p = 0.001$ ), Australia ( $p < 0.001$ ) and South Africa ( $p < 0.001$ ). The prevalence of CMt

1 infection found in the present study was not significantly different to that reported in  
2 samples from the UK and Switzerland but was significantly lower than that reported  
3 in samples from Australia ( $p < 0.001$ ) and South Africa ( $p < 0.001$ ).

4 Infections with both single and multiple haemoplasma species were detected,  
5 with 30 (1.9%), 154 (9.7%) and 13 (0.8%) samples positive for Mhf, CMhm and CMt  
6 alone (Table 5). Twenty five (1.6%) samples were positive for more than one  
7 haemoplasma species: eleven (0.7%) Mhf/CMhm, two (0.1%) Mhf/CMt, and ten  
8 (0.6%) CMhm/CMt dual infections as well as two (0.1%) infections with all three  
9 species. The species detected in the blood samples with multiple infections were  
10 present at differing copy numbers with a minimum of a ten-fold difference between  
11 the species in the dual infections and, with the exception of one Mhf/CMt infection,  
12 CMt was the species present at the lowest copy number in all dual and triple  
13 infections involving this organism. Two different species were dominant in the two  
14 triple infections: either Mhf or CMhm.

## 15 **Discussion**

16 The results of this study show that it is possible to develop feline  
17 haemoplasma species-specific qPCR assays, based upon 16S rDNA sequences,  
18 duplexed with an internal control assay based upon feline 28S rDNA, which are  
19 highly sensitive. This is the first description of this type of assay for the diagnosis of  
20 feline haemoplasma infection as those described previously (Tasker et al., 2003b;  
21 Willi et al., 2006a; Willi et al., 2005) have not included an internal control assay,  
22 although some studies using these assays have used an internal control as a separate  
23 PCR. The advantage of using an internal control assay is that it allows for  
24 confirmation that the test sample contains amplifiable DNA and that PCR inhibitors

1 are not present. In addition, using the internal control assay in a duplex allows  
2 confirmation that the sample has been added to the reaction. Blood contains a number  
3 of potential inhibitors of PCR (Al-Soud and Radstrom, 2001) and although some,  
4 such as haemoglobin, may be visible in the extracted DNA, others may be colourless  
5 and their presence impossible to detect without use of an internal control assay.  
6 Haemoglobin contamination can be a significant problem with DNA obtained from  
7 feline blood samples, particularly if there is a blood clot present in the sample  
8 (unpublished observation). Seven samples were excluded from the study on the basis  
9 of their 28S rDNA PCR results and it is of note that these samples had been submitted  
10 for diagnostic PCR testing prior to the introduction of the 28S rDNA internal control  
11 assay into the haemoplasma diagnostic assays. Thus it is possible that these samples  
12 generated false negative haemoplasma PCR results previously but the lack of use of  
13 an internal control precluded this being detected.

14         The qPCR assays described in this study were able to detect between 1 and 10  
15 copies per PCR in the presence of a background of feline DNA similar to that found  
16 in the extracted blood samples. No attempt was made to examine the effect of the  
17 increasing the concentration of feline DNA on the ability of the assays to detect the  
18 haemoplasma species. This was because a fixed volume of 100µl of EDTA blood is  
19 used for the extraction process and this volume results in a relatively constant amount  
20 of feline genomic DNA in the extracted samples. It is possible that the sensitivity of  
21 the haemoplasma portion of the duplex PCR may have been affected by a larger  
22 amount of 28S rDNA, and therefore feline genomic DNA, which may be present if  
23 samples other than blood e.g. tissue, were used instead. There was evidence that the  
24 28S rDNA results were affected by the presence of high 16S rDNA copy numbers (in  
25 excess of  $10^6$ /reaction), with evidence of inhibition of the reaction proportional to the

1 amount of 16S rDNA present. This was manifest by a higher 28S rDNA Ct value in  
2 the assay which was haemoplasma PCR positive when compared with the assays with  
3 negative haemoplasma results. It would have been feasible to have combined the  
4 three haemoplasma 16S rDNA assays with the feline 28S rDNA assay to form a  
5 quadriplex assay by changing the fluorescent dyes on two of the haemoplasma  
6 TaqMan probes. We decided not to do this as infections with multiple species have  
7 been reported and there may be a dominant species present which could affect the  
8 sensitivity of the assays for the other species in the quadriplex assay, similar to the  
9 phenomenon seen with the 28S rDNA assay in the duplex assays.

10 The qPCR assays developed in this study replaced the previously described  
11 qPCR assays (Tasker et al., 2003b) for the diagnosis of haemoplasma infection by  
12 Langford Veterinary Diagnostics in April 2005. Prior to this, no samples were  
13 identified as being positive for multiple species, although 18/25 samples identified in  
14 the present study with multiple haemoplasma species had been assayed prior to this  
15 date and, therefore, with the previous qPCR assays. The exact qPCR results  
16 previously obtained from these 18 samples is unknown as the samples were  
17 anonymised but they were identified as 10 Mhf/CMhm, one Mhf/CMt and 5  
18 CMhm/CMt dual infected and two triple infected samples when tested with the new  
19 assays. The previously described qPCR assay (Tasker et al., 2003b) does not detect  
20 CMt (although the primers used in this assay are capable of producing a PCR  
21 amplicon of similar size to Mhf) because neither of the two TaqMan probes used in  
22 the previous qPCR assay are capable of hybridising to the product amplified by the  
23 primers from CMt template (Jensen et al., 2001; Willi et al., 2005). This fact explains  
24 the failure to detect the dual infected samples with the previous assays where CMt  
25 was one of the organisms present. The failure of the previous qPCR assay to identify

1 the 12 (both dual and triple infected) samples infected with both Mhf and CMhm may  
2 be due to one of the species being dominant in the reaction and thus inhibiting  
3 amplification of the minor species through competition for reaction components  
4 (Tasker, 2002). This phenomenon is a potential problem with assays, like those  
5 previously described for detection of Mhf and CMhm (Sykes et al., 2007; Tasker et  
6 al., 2003b; Willi et al., 2006a, ), which rely solely on the TaqMan probes to  
7 differentiate the species present in the sample following amplification with universal  
8 primers rather than on species-specific primers and probes. This phenomenon would  
9 not be a problem with the previously described assay for the detection of CMt (Willi  
10 et al., 2005) which uses species-specific primers and probes. The assays we  
11 developed in this study do not amplify non-target species and therefore the results  
12 from one haemoplasma species will not affect the results of the others, therefore  
13 maximising the chances of detecting all of the three species present in a sample  
14 despite differing copy numbers.

15 The prevalences of the three haemoplasma species identified in the present  
16 study are very similar to those found in recent studies of cats from Switzerland (Willi  
17 et al., 2006a) and the UK (Tasker et al., 2003a; Willi et al., 2006b) with the exception  
18 of the prevalence of CMhm which was significantly lower in the present study than  
19 that found previously in UK samples. The samples in the present study were derived  
20 from those submitted to a diagnostic service for haemoplasma testing and are likely  
21 composed primarily of cats which are anaemic or suspected of having haemoplasma  
22 infection due to results from other diagnostic tests with only a very small number of  
23 samples from cats being screened prior to use as blood donors. The samples used in  
24 the study of cats from the UK were collected from both healthy and sick cats (Tasker  
25 et al., 2003a; Willi et al., 2006b) and may therefore not be equivalent to those used in

1 the present study. The lower prevalence of CMhm may be due to a smaller number of  
2 samples from cats infected with this species being submitted to the diagnostic service  
3 because of the milder clinical signs produced by this species in the absence of  
4 concurrent infections or immunosuppression (De Lorimier and Messick, 2004; Foley  
5 et al., 1998; George et al., 2002). This could result in a lower prevalence of infection  
6 in our study group compared with the one which included a larger number of  
7 apparently healthy cats (Tasker et al., 2003a).

8 The prevalence of all three haemoplasma species in the present study was  
9 significantly lower than that reported in cats from South Africa (Lobetti and Tasker,  
10 2004; Willi et al., 2006b), as was the prevalence of CMhm and CMt when compared  
11 to cats from Australia (Tasker et al., 2004; Willi et al., 2006b). The South African  
12 and Australian study samples were mostly collected from cats which were clinically  
13 ill and therefore likely derive from a similar cat population as the samples used in this  
14 study.

15 A large number of infections involving multiple organisms were identified in  
16 the current study. Infection with CMt is often associated with infection with other  
17 haemoplasma species, particularly CMhm, as has been reported previously (Willi et  
18 al., 2006b). We have also described two samples which were infected with all three  
19 species; one with a Mhf-dominated infection and the other with a CMhm-dominated  
20 one. Similar triple infections have been described in two samples from South Africa  
21 (Willi et al., 2006b). Interestingly, CMt is often the organism which is present at the  
22 lowest copy number in dual and triple infections, as has been reported previously  
23 (Willi et al., 2006a; Willi et al., 2006b). Increased severity of anaemia has been  
24 associated with co-infection with CMt and either CMhm or Mhf (Willi et al., 2006b)  
25 but additional haematological and clinical data were not available for the samples

1 analysed in this study and, therefore, the effect that co-infection with CMt on the  
2 severity of anaemia associated with CMhm and Mhf infection could not be assessed.  
3 The presence of CMt at lower copy numbers highlights the importance of diagnostic  
4 assays which are not affected by the presence of other haemoplasma species in order  
5 to prevent false negative results involving this organism.

6 The results of this study demonstrate that it is possible to develop species-  
7 specific 16S rDNA-based qPCR assays duplexed with a feline 28S rDNA assay for  
8 the diagnosis of feline haemoplasma infection. The inclusion of an internal control  
9 assay is important in order to prevent false negative results due to the failure of DNA  
10 extraction or the presence of PCR inhibitors. As infections with multiple  
11 haemoplasma species are common, it is important to have assays which are able to  
12 detect low levels of the target haemoplasma species in the presence of high levels of  
13 non-target species. This was achieved by designing novel haemoplasma assays which  
14 utilised individual species-specific primers and probes rather than universal  
15 haemoplasma primers and species-specific probes. The most commonly identified  
16 infection was with CMhm, and CMt infection was frequently associated with co-  
17 infection with other haemoplasma species, especially CMhm.

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- 3

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1 **Table 1: Details of the quantitative PCR assays used for the detection of the three haemoplasma species and feline 28S rDNA.**

Target	Forward Primer	Reverse Primer	5' Fluorophore	Probe	3' Quencher	Product Size (base pairs)	Melting Temperature (°C)	Reaction Efficiency (%)	R <sup>2</sup>
<b>Mhf</b>	GTGCTACAATGG CGAACACA	TCCTATCCGAAC GAGACGAA	FAM	TGTGTTGCAAACCAG CGATGGT	BHQ-1	80	84.0	99.1	1.00
<b>CMhm</b>	TGATCTATTGTKA AAGGCACTTGCT	TTAGCCTCYGGTG TTCCTCAA	FAM	TTCAATGTGTAGCGG TGGAATGCGT	BHQ-1	135	84.5	93.0	0.99
<b>CMt</b>	AGAGGCGAAGGC GAAAAC	CTACAACGCCGA AACACAAA	FAM	CGTAAACGATGGGTA TTAGATGTCGGGAT	BHQ-1	138	87.0	91.3	1.00
<b>Feline 28S rDNA</b>	AGCAGGAGGTGT TGGAAGAG	AGGGAGAGCCTA AATCAAAGG	Texas Red	TGGCTTGTGGCAGCC AAGTGT	BHQ-2	100			

2 Mhf: *Mycoplasma haemofelis*, CMhm: '*Candidatus Mycoplasma haemominutum*', CMt: '*Candidatus Mycoplasma turicensis*', R<sup>2</sup>:

3 correlation coefficient

1 **Table 2: Alignment of primer and probe sequences with target and non-target species 16S rDNA haemoplasma genes**

	<b>Forward Primer 5'-3'</b>	<b>Probe 5'FAM-Probe-3' BHQ-1</b>	<b>Reverse Primer 5'-3'</b>
<b>Mhf</b>	GTGCTACAATGGCGAACACA	TGTGTTGCAAACCAGCGATGGT	TCCTATCCGAACTGAGACGAA
<b>CMhm</b>	-----TAGG--T-	----C----T-T--AA--A-G	-TT-----G-----TAGG
<b>CMt</b>	---T-----T--GT---	----C--G-----	-TT-----G-----T--G
<b>Mhf</b>	A-T---G- -----T---GC-A--	--A-GCA--G-----T--	--C-----T-AG
<b>CMhm</b>	TGATCTATTGTKAAAGGCACTTGCT	TTCAATGTGTAGCGGTGGAATGCGT	TTAGCCTCYGGTGTTCCTCAA
<b>CMt</b>	A-T---GC A-----A--GC----	--A--A--G-----T--	--C-----T-A--
<b>Mhf</b>	-----G-----	-----A-TA--GC	-----A-TA--G-TA--G
<b>CMhm</b>	-----T-----GT-TC	-----G-A-TT-TC	-----G-A-TC-GT- -T-G
<b>CMt</b>	AGAGGCGAAGGCGAAAAC	CGTAAACGATGGGTATTAGATGTCGGGAT	CTACAACGCCGAAACACAAA

2 Mhf: *Mycoplasma haemofelis*, CMhm: '*Candidatus Mycoplasma haemominutum*', CMt: '*Candidatus Mycoplasma turicensis*'

- 1 **Table 3: Number of haemoplasma PCR-positive samples categorized by 16S rDNA gene copy numbers in the 1585 feline blood samples**  
 2 **with adequate 28S rDNA analysed in the study**

Haemoplasma species	Number of Samples Positive for Species (n=)				Total Number (% Samples Tested)
	1-100 copies per PCR	100-10 <sup>4</sup> copies per PCR	10 <sup>4</sup> -10 <sup>6</sup> copies per PCR	greater than 10 <sup>6</sup> copies per PCR	
<b>Mhf</b>	20 (44.4)	11 (24.4)	6 (13.4)	8 (17.8)	45 (2.8)
<b>CMhm</b>	37 (20.9)	64 (36.2)	70 (39.5)	6 (3.4)	177 (11.2)
<b>CMt</b>	9 (33.3)	11 (40.7)	6 (22.2)	1 (3.7)	27 (1.7)
<b>Negative</b>					1336 (84.3)

- 3 Mhf: *Mycoplasma haemofelis*, CMhm: ‘*Candidatus Mycoplasma haemominutum*’, CMt: ‘*Candidatus Mycoplasma turicensis*’

1 **Table 4: Number and percentage (sample prevalence) of cat blood samples positive for the three haemoplasma species<sup>a</sup>**

Sample Source (n=)	Number of Positive Samples (% of Samples Tested $\pm$ 95% CI)		
	Mhf	CMhm	CMt
<b>UK Current Study (1585)</b>	45 (2.8 $\pm$ 0.8)	177 (11.2 $\pm$ 1.6)	27 (1.7 $\pm$ 0.6)
<b>UK (426)</b>	7 (1.6 $\pm$ 1.2)	73 (17.1 $\pm$ 3.6)	10 (2.3 $\pm$ 1.4)
<b>Switzerland (713)</b>	11 (1.5 $\pm$ 0.9)	71 (10.0 $\pm$ 1.2)	9 (1.3 $\pm$ 0.8)
<b>Australia (147)</b>	7 (4.8 $\pm$ 3.4)	35 (23.8 $\pm$ 6.9)	15 (10.2 $\pm$ 4.9)
<b>South Africa (69)<sup>b</sup></b>	10 (14.5 $\pm$ 8.2)	26 (37.7 $\pm$ 11.4)	18 (26.1 $\pm$ 10.3)

2 Mhf: *Mycoplasma haemofelis*, CMhm: ‘*Candidatus Mycoplasma haemominutum*’, CMt: ‘*Candidatus Mycoplasma turicensis*’

3 <sup>a</sup> Results of recent PCR-based studies (Lobetti and Tasker, 2004; Tasker et al., 2003a; Tasker et al., 2004; Willi et al., 2006a; Willi et al., 2006b)  
 4 investigating the prevalence of the three haemoplasma species are included for comparison. The 95% CI were calculated for the published data.

5 <sup>b</sup> Only the results of the 69 samples tested for CMt in the study by Willi *et al.*(2006b) were included in the calculations. Nine of the original 78  
 6 samples tested for Mhf and CMhm by Lobetti *et al.*(2004) were not assayed for CMt.

1 **Table 5: Number and percentage (sample prevalence) of haemoplasma single, double and triple infections in the present study<sup>a</sup>**

Sample Source (n=)	Number of Positive Samples (% of Samples Tested $\pm$ 95% CI)						
	Mhf Only	CMhm Only	CMt Only	Mhf CMhm	Mhf CMt	CMhm CMt	Mhf CMhm CMt
<b>UK Current Study (1585)</b>	30 (1.9 $\pm$ 0.7)	154 (9.7 $\pm$ 1.5)	13 (0.8 $\pm$ 0.4)	11 (0.7 $\pm$ 0.4)	2 <sup>b</sup> (0.1)	10 (0.6 $\pm$ 0.4)	2 <sup>b</sup> (0.1)
<b>UK (426)</b>	5 (1.2 $\pm$ 1.0)	66 (15.5 $\pm$ 3.4)	3 <sup>b</sup> (0.7)	1 <sup>b</sup> (0.2)	1 <sup>b</sup> (0.2)	6 (1.4 $\pm$ 1.1)	0
<b>Switzerland (713)</b>	9 (1.3 $\pm$ 0.8)	63 (8.8 $\pm$ 2.1)	3 <sup>b</sup> (0.4)	2 <sup>b</sup> (0.3)	0	6 (0.8 $\pm$ 0.7)	0
<b>Australia (147)</b>	6 (4.1 $\pm$ 3.2)	26 (17.7 $\pm$ 6.2)	6 (4.1 $\pm$ 3.2)	1 <sup>b</sup> (0.7)	1 <sup>b</sup> (0.7)	8 (5.4 $\pm$ 3.7)	0
<b>South Africa (69)<sup>c</sup></b>	1 <sup>b</sup> (1.4)	10 (14.5 $\pm$ 8.3)	7 (10.1 $\pm$ 7.1)	5 (7.2 $\pm$ 6.1)	2 <sup>b</sup> (2.9)	9 (13.0 $\pm$ 7.9)	2 <sup>b</sup> (2.9)

2 Mhf: *Mycoplasma haemofelis*, CMhm: ‘*Candidatus Mycoplasma haemominutum*’, CMt: ‘*Candidatus Mycoplasma turicensis*’

3 <sup>a</sup> Results of recent PCR-based studies (Lobetti and Tasker, 2004; Tasker et al., 2003a; Tasker et al., 2004; Willi et al., 2006a; Willi et al., 2006b)  
4 investigating the prevalence of the three haemoplasma species are included for comparison. The 95% CI were calculated for the published data.

5 <sup>b</sup> 95% CI were not calculated for these results due to the small number of positive samples

6 <sup>c</sup> Only the results of the 69 samples tested for CMt in the study by Willi *et al.*(2006b) were included in the calculations. Nine of the original 78  
7 samples tested for Mhf and CMhm by Lobetti *et al.*(2004) were not assayed for CMt.

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