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

Iain R. Peters¹, Chris R. Helps¹, Barbara Willi², Regina Hofmann-Lehmann², Séverine
Tasker^{1*}

¹ School of Clinical Veterinary Science, University of Bristol, UK
² Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Switzerland

Corresponding Author:

Dr Iain Peters

School of Clinical Veterinary Science

University of Bristol

Langford House

Langford

Bristol

BS40 5DU

UK

Tel: + 44 (0)117 928 9690

Fax: + 44 (0)117 928 9588

i.r.peters@bristol.ac.uk

- 1 KEYWORDS: Feline Anaemia, Feline Haemoplasma, Quatitative Real-time PCR,
- 2 Diagnosis-Bacterial

Abstract

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

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

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

Materials and Methods

Primer and Probe Design

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

Samples and DNA Extraction

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

Real-time PCR (qPCR)

Real-time PCR was performed using Qiagen HotStarTaq Master Mix (Crawley, UK) with 200 nM of each primer, 100 nM of each probe or SYBR Green 1 (1:100,000 final dilution) (Sigma-Aldrich Ltd., Poole, Dorset), 4.5 mM MgCl₂ and 5 µl of DNA in a total volume of 25µl. All reactants were mixed together as a master mix and aliquotted into 24 or 96-well PCR plates (Thermofast, Abgene, Epsom, Surrey) prior to addition of the 5µl sample. Positive control samples of known copy number and a negative control (water) were included in each run.

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

Previously sequenced plasmids containing the entire 16S rDNA gene from each of the target species (Tasker et al., 2003c; Willi et al., 2005) were used to test the efficiency and specificity of the reactions. A ten-fold serial dilution of each plasmid (range: 1 to 10⁷ copies/reaction), mixed with an equal volume of genomic DNA extracted from 200µl of EDTA blood from a specific pathogen free cat, was used to calculate the reaction efficiency for each of the haeomplasma-specific assays. A

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

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

Statistical Analysis

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

Results

Assay Optimisation

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

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

Clinical Samples

The number of haemoplasma PCR-positive samples and the 16S rDNA copy numbers for each haemoplasma species are shown in Table 3. All of the 222 haemoplasma positive samples were also positive for feline 28S rDNA, although the 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

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

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

Discussion

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

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

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

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

The prevalences of the three haemoplasma species identified in the present study are very similar to those found in recent studies of cats from Switzerland (Willi et al., 2006a) and the UK (Tasker et al., 2003a; Willi et al., 2006b) with the exception of the prevalence of CMhm which was significantly lower in the present study than that found previously in UK samples. The samples in the present study were derived from those submitted to a diagnostic service for haemoplasma testing and are likely composed primarily of cats which are anaemic or suspected of having haemoplasma infection due to results from other diagnostic tests with only a very small number of samples from cats being screened prior to use as blood donors. The samples used in the study of cats from the UK were collected from both healthy and sick cats (Tasker 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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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 ²
Mhf	GTGCTACAATGG CGAACACA	TCCTATCCGAAC GAGACGAA	FAM	TGTGTTGCAAACCAG CGATGGT	BHQ-1	80	84.0	99.1	1.00
CMhm	TGATCTATTGTGA AAGGCACTTGCT	TTAGCCTCYGGTG TTCCTCAA	FAM	TTCAATGTGTAGCGG TGGAATGCGT	BHQ-1	135	84.5	93.0	0.99
CMt	AGAGGCGAAGGC GAAAACT	CTACAACGCCGA AACACAAA	FAM	CGTAAACGATGGGTA TTAGATGTCGGGAT	BHQ-1	138	87.0	91.3	1.00
Feline 28S rDNA	AGCAGGAGGTGT TGGAAGAG	AGGGAGAGCCTA AATCAAAGG	Texas Red	TGGCTTGTGGCAGCC AAGTGT	BHQ-2	100			

2 Mhf: *Mycoplasma haemofelis*, CMhm: '*Candidatus* Mycoplasma haemominutum', CMt: '*Candidatus* Mycoplasma turicensis', R²:

3 correlation coefficient

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

	Forward Primer 5'-3'	Probe 5'FAM-Probe-3' BHQ-1	Reverse Primer 5'-3'
Mhf	GTGCTACAATGGCGAACACA	TGTGTTGCAAACCAGCGATGGT	TCCTATCCGAACTGAGACGAA
CMhm	-----TAGG--T-	----C---T-T--AA--A-G	-TT-----G-----TAGG
CMt	---T-----T--GT---	----C--G-----	-TT-----G-----T--G
Mhf	A-T---G- -----T---GC-A--	--A-GCA--G-----T--	--C-----T-AG
CMhm	TGATCTATTGTCAAAGGCACTTGCT	TTCAATGTGTAGCGGTGGAATGCGT	TTAGCCTCYGGTGTTCCTCAA
CMt	A-T---GC A-----A---GC----	--A---A--G-----T--	--C-----T-A--
Mhf	-----G-----	-----A-TA--GC	-----A-TA--G-TA--G
CMhm	-----T-----GT-TC	-----G-A-TT-TC	-----G-A-TC-GT- -T-G
CMt	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=) (% of Total Species Positive Samples)				Total Number (% Samples Tested)
	1-100 copies per PCR	100-10 ⁴ copies per PCR	10 ⁴ -10 ⁶ copies per PCR	greater than 10 ⁶ copies per PCR	
Mhf	20 (44.4)	11 (24.4)	6 (13.4)	8 (17.8)	45 (2.8)
CMhm	37 (20.9)	64 (36.2)	70 (39.5)	6 (3.4)	177 (11.2)
CMt	9 (33.3)	11 (40.7)	6 (22.2)	1 (3.7)	27 (1.7)
Negative					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^a**

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

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
UK Current Study (1585)	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 ^b (0.1)	10 (0.6 \pm 0.4)	2 ^b (0.1)
UK (426)	5 (1.2 \pm 1.0)	66 (15.5 \pm 3.4)	3 ^b (0.7)	1 ^b (0.2)	1 ^b (0.2)	6 (1.4 \pm 1.1)	0
Switzerland (713)	9 (1.3 \pm 0.8)	63 (8.8 \pm 2.1)	3 ^b (0.4)	2 ^b (0.3)	0	6 (0.8 \pm 0.7)	0
Australia (147)	6 (4.1 \pm 3.2)	26 (17.7 \pm 6.2)	6 (4.1 \pm 3.2)	1 ^b (0.7)	1 ^b (0.7)	8 (5.4 \pm 3.7)	0
South Africa (69)^c	1 ^b (1.4)	10 (14.5 \pm 8.3)	7 (10.1 \pm 7.1)	5 (7.2 \pm 6.1)	2 ^b (2.9)	9 (13.0 \pm 7.9)	2 ^b (2.9)

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

3 ^a 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 ^b 95% CI were not calculated for these results due to the small number of positive samples

6 ^c 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.

References

- Al-Soud, W.A., Radstrom, P., 2001, Purification and characterization of PCR-inhibitory components in blood cells. *J Clin Microbiol* 39, 485-493.
- Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003, Presence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study. *Vet Microbiol* 93, 307-317.
- De Lorimier, L.P., Messick, J.B., 2004, Anemia Associated With 'Candidatus *Mycoplasma haemominutum*' in a Feline Leukemia Virus-Negative Cat With Lymphoma. *J Am Anim Hosp Assoc* 40, 423-427.
- Foley, J.E., Harrus, S., Poland, A., Chomel, B., Pedersen, N.C., 1998, Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats. *Am J Vet Res* 59, 1581-1588.
- Foley, J.E., Pedersen, N.C., 2001, 'Candidatus *Mycoplasma haemominutum*', a low-virulence epierthrocytic parasite of cats. *Int J Syst Evol Microbiol* 51, 815-817.
- George, J.W., Rideout, B.A., Griffey, S.M., Pedersen, N.C., 2002, Effect of preexisting FeLV infection or FeLV and feline immunodeficiency virus coinfection on pathogenicity of the small variant of *Haemobartonella felis* in cats. *Am J Vet Res* 63, 1172-1178.
- Helps, C.R., Lait, P., Damhuis, A., Bjornehammar, U., Bolta, D., Brovida, C., Chabanne, L., Egberink, H., Ferrand, G., Fontbonne, A., Pennisi, M.G., Gruffydd-Jones, T., Gunn-Moore, D., Hartmann, K., Lutz, H., Malandain, E., Mostl, K., Stengel, C., Harbour, D.A., Graat, E.A., 2005, Factors associated with upper respiratory tract disease caused by feline herpesvirus, feline calicivirus, *Chlamydophila felis* and *Bordetella bronchiseptica* in cats: experience from 218 European catteries. *Vet Rec* 156, 669-673.
- Jensen, W.A., Lappin, M.R., Kamkar, S., Reagan, W.J., 2001, Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats. *Am J Vet Res* 62, 604-608.
- Lobetti, R.G., Tasker, S., 2004, Diagnosis of feline haemoplasma infection using a real-time PCR assay. *J S Afr Vet Assoc* 75, 94-99.

- 1 Luria, B.J., Levy, J.K., Lappin, M.R., Breitschwerdt, E.B., Legendre, A.M.,
2 Hernandez, J.A., Gorman, S.P., Lee, I.T., 2004, Prevalence of infectious
3 diseases in feral cats in Northern Florida. *J Feline Med Surg* 6, 287-296.
- 4 Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 2001, Proposal to transfer
5 some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus
6 *Mycoplasma* with descriptions of '*Candidatus Mycoplasma haemofelis*',
7 '*Candidatus Mycoplasma haemomuris*', '*Candidatus Mycoplasma haemosuis*'
8 and '*Candidatus Mycoplasma wenyonii*'. *Int J Syst Evol Microbiol* 51, 891-
9 899.
- 10 Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 2002, Revision of
11 haemotrophic *Mycoplasma* species names. *Int J Syst Evol Microbiol* 52, 683.
- 12 Sykes, J.E., Drazenovich, N.L., Kyles, A.E., Ball, L.M., Leutenegger, C.M., 2007,
13 Detection of mixed infections with "*Candidatus Mycoplasma haemominutum*"
14 and *Mycoplasma haemofelis* using real-time TaqMan polymerase chain
15 reaction. *J Vet Diagn Invest* 19, 250-255.
- 16 Tasker, S., 2002. Feline Haemoplasmas- Detection, Infection, Dynamics and
17 Distribution. PhD Thesis. University of Bristol, Bristol, UK.
- 18 Tasker, S., Binns, S.H., Day, M.J., Gruffydd-Jones, T.J., Harbour, D.A., Helps, C.R.,
19 Jensen, W.A., Olver, C.S., Lappin, M.R., 2003a, Use of a PCR assay to assess
20 the prevalence and risk factors for *Mycoplasma haemofelis* and '*Candidatus*
21 *Mycoplasma haemominutum*' in cats in the United Kingdom. *Vet Rec* 152,
22 193-198.
- 23 Tasker, S., Braddock, J.A., Baral, R., Helps, C.R., Day, M.J., Gruffydd-Jones, T.J.,
24 Malik, R., 2004, Diagnosis of feline haemoplasma infection in Australian cats
25 using a real-time PCR assay. *J Feline Med Surg* 6, 345-354.
- 26 Tasker, S., Helps, C.R., Day, M.J., Gruffydd-Jones, T.J., Harbour, D.A., 2003b, Use
27 of real-time PCR to detect and quantify *Mycoplasma haemofelis* and
28 "*Candidatus Mycoplasma haemominutum*" DNA. *J Clin Microbiol* 41, 439-
29 441.
- 30 Tasker, S., Helps, C.R., Day, M.J., Harbour, D.A., Shaw, S.E., Harrus, S., Baneth, G.,
31 Lobetti, R.G., Malik, R., Beauvils, J.P., Belford, C.R., Gruffydd-Jones, T.J.,
32 2003c, Phylogenetic analysis of hemoplasma species: an international study. *J*
33 *Clin Microbiol* 41, 3877-3880.

- 1 Willi, B., Boretti, F.S., Baumgartner, C., Tasker, S., Wenger, B., Cattori, V., Meli,
2 M.L., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2006a, Prevalence, risk
3 factor analysis, and follow-up of infections caused by three feline hemoplasma
4 species in cats in Switzerland. J Clin Microbiol 44, 961-969.
- 5 Willi, B., Boretti, F.S., Cattori, V., Tasker, S., Meli, M.L., Reusch, C., Lutz, H.,
6 Hofmann-Lehmann, R., 2005, Identification, molecular characterization, and
7 experimental transmission of a new hemoplasma isolate from a cat with
8 hemolytic anemia in Switzerland. J Clin Microbiol 43, 2581-2585.
- 9 Willi, B., Tasker, S., Boretti, F.S., Doherr, M.G., Cattori, V., Meli, M.L., Lobetti,
10 R.G., Malik, R., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2006b,
11 Phylogenetic Analysis of "Candidatus Mycoplasma turicensis" Isolates from
12 Pet Cats in the United Kingdom, Australia, and South Africa, with Analysis of
13 Risk Factors for Infection. J Clin Microbiol 44, 4430-4435.
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