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SHORT COMMUNICATION
Use of real-time PCR to detect *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' in the saliva and salivary glands of haemoplasma-infected cats

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Feline haemoplasma infection can cause haemolytic anaemia. The natural method of transmission of haemoplasmas between cats is currently unknown but the nature of some of the risk factors for infection suggests that saliva may act as a mode of transmission. The aim of this study was to determine if *Mycoplasma haemofelis* (Mhf) and '*Candidatus* Mycoplasma haemominutum' (CMhm) DNAs could be amplified from saliva and salivary gland samples collected from haemoplasma-infected cats.

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Three distinct species of feline haemoplasma have been described: *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt) (Foley and Pedersen 2001, Neimark et al 2001, 2002, Willi et al 2005). These epierythrocytic bacteria can cause anaemia via induction of haemolysis. The different species demonstrate varying degrees of pathogenicity with CMhm being the least pathogenic species (Foley and Pedersen 2001).

The natural mode of transmission of haemoplasmas is not known. Infection has been achieved experimentally using infected blood via intravenous, intraperitoneal and oral inoculation routes. Haemoplasma DNA has been identified in *Ctenocephalides felis* and transmission of Mhf via haemophagous activity has been demonstrated experimentally (Lappin et al 2003, Shaw et al 2004, Woods et al 2005). The risk factors for haemoplasma infection that have been described in the veterinary literature include male gender, increasing age, feline immunodeficiency virus infection, outdoor roaming and a recent history of a cat bite abscesses (Grindem et al 1990, Tasker et al 2003a, Luria et al 2004, Tasker et al 2004). The nature of these risk factors suggests that haemoplasma infection might be transmitted via saliva during cat bites and the presence of CMt in the saliva of CMt-infected cats has been reported (Willi et al 2006).

To test this hypothesis a study was undertaken to ascertain if haemoplasma DNA could be identified in the saliva and/or salivary glands of haemoplasma-infected cats.

Saliva samples were obtained from cats experimentally infected with either CMhm (six cats; S1–S6) or Mhf (four cats; S7–S10) at 79 days post-infection (pi), and from two uninfected control cats (S11 and S12). Quantitative real-time polymerase chain reaction (PCR) (Tasker et al 2003b), performed on blood samples collected from all 12 cats on the day of saliva collection, was positive in all 10 haemoplasma-infected cats and negative in the two uninfected control

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cats. Saliva was collected by placing a cotton wool-tipped swab sublingually and in the fauces of the mouth of each cat for 30 s. The swabs were centrifuged (8000 *g* for 3 min) to elute both the saliva, and exfoliated cells (as a pellet), from the swab. The saliva supernatant was aspirated (approximately 300−500 µl per sample) from the cell pellet and the swab. The swab, saliva supernatant and cell pellet from each cat were stored individually at −80°C until analysis. Two hundred microlitres of buffer B3 and 20 µl proteinase K were added to each of the three fractions prior to DNA extraction: a 200 µl sample of saliva supernatant, the cell pellet suspended in 200 µl of phosphate buffered saline (PBS) and the swab suspended in 200 µl of PBS. DNA was extracted from each fraction using the Nucleospin Blood kit (Macherey−Nagel, Germany) according to the manufacturer's instructions and eluted into 100 µl of buffer BE.

Salivary gland tissue was obtained at necropsy from five cats infected with CMhm at 153 days pi (cats; G1−G5) and from three cats infected with Mhf at 118 days pi (cats; G6−G8). Quantitative real-time PCR (Tasker et al 2003b), performed on blood samples from each cat collected at the time of salivary gland collection, were positive for the infecting haemoplasma species. DNA was extracted, using the Nucleospin Blood kit (Macherey−Nagel, Germany) according to the manufacturer's instructions, from 20 mg of tissue using 180 µl of TI buffer and 20 µl of proteinase K with incubation at 56°C overnight. DNA was eluted into 100 µl of buffer BE.

Five microlitres of DNA extracted from each sample (saliva supernatant, cell pellet, swab and salivary gland) was then subjected to quantitative real-time PCR (Tasker et al 2003b). Samples extracted from known haemoplasma-infected cats and water were used as positive and negative controls, respectively.

A further aliquot of DNA was subjected to a real-time PCR assay for feline 28S rDNA, to ensure that samples contained amplifiable DNA. The PCR mixture consisted of 12.5 µl of 2X Hotstartaq mastermix (Qiagen); 200 nM of the 28S forward (5′-AGCAGGAGGTGTTGGAAGAG-3′) and reverse (5′-AGGGAGAGCCTAAATCAAAGG-3′) primers (Cruachem, Glasgow, Scotland); 100 nM of 28S Taqman probe (5′Texas Red-TGGCTTGTGGCAGCCAAGTGT-BHQ2-3′) (Cruachem); $MgCl_2$ to a final concentration of 4.5 mM; and 5 µl of template DNA made up to a final volume of 25 µl with water. After an initial incubation at 95°C for 15 min to activate the Hotstartaq polymerase, 45 cycles of 10 s at 95°C and 30 s at 60°C were performed. Samples from known 28S rDNA positive samples and water were used as positive and negative controls, respectively.

The copy numbers of CMhm and Mhf present in the blood of the haemoplasma-infected cats used in the current study varied greatly between individuals as shown in Tables 1 and 2.

Table 1 summarises the saliva sampling results obtained. No gross blood contamination was evident in any of the collected saliva samples. All 12 swabs, nine of the 12 saliva supernatant samples and 10 of the 12 cell pellets were PCR positive for 28S rDNA. Low levels (1−100 copies per PCR) of CMhm DNA were detected in the saliva and cell pellet of one cat (cat S4) and the swab of another (cat S2) out of a possible six CMhm-infected cats (Table 1). Mhf DNA was not detected in any of the saliva samples collected from the four Mhf-infected cats.

Table 2 summarises the salivary gland sampling results obtained. All salivary gland samples were positive for 28S rDNA. Low levels (1−100 copies per PCR) of CMhm DNA were detected in two (G3 and G5) of five salivary gland samples from CMhm-infected cats. Mhf was not detected in any of the salivary gland samples collected from the three Mhf-infected cats (Table 2).

'*Candidatus* M haemominutum' DNA, but not Mhf DNA, was detectable in the saliva and salivary glands of some CMhm-infected cats, supporting the hypothesis that saliva may act as a route of CMhm transmission, as has been previously postulated for CMt (Willi et al 2006).

The number of haemoplasma DNA copies present in the blood of the cats at the time of saliva sampling was very variable. In the Mhf-infected cats this was likely due to previous antibiotic treatment and the cyclical variation in copy number seen during the course of Mhf infection (Tasker et al 2006b). The CMhm-infected cats tended to have higher numbers of haemoplasma DNA copies than the Mhf-infected cats as previous antibiotic treatment did not have a major effect on CMhm copy numbers and cyclical variation has not been observed in CMhm-infected cats (Tasker et al 2006a). The lower blood copy numbers found in the Mhf-infected cats may have contributed to the failure to amplify Mhf in the saliva and salivary gland samples obtained from Mhf-infected cats. Additionally, the marked variation in copy number that can occur in Mhf-infected cats may influence the presence of

Table 1. Saliva sampling results

Cat ID	Infecting haemoplasma species	Blood haemoplasma DNA copy number (per μl of blood)	Saliva supernatant		Swab		Cell pellet	
			Haemoplasma DNA	28S rDNA	Haemoplasma DNA	28S rDNA	Haemoplasma DNA	28S rDNA
S1	CMhm	273,063	−	+	−	+	−	−
S2	CMhm	22,879	−	+	+	+	−	+
S3	CMhm	208,857	−	−	−	+	−	+
S4	CMhm	114,266	+	+	−	+	+	+
S5	CMhm	66,848	−	−	−	+	−	+
S6	CMhm	22,333	−	+	−	+	−	+
S7	Mhf	7532	−	+	−	+	−	+
S8	Mhf	5	−	+	−	+	−	+
S9	Mhf	41,210	−	+	−	+	−	−
S10	Mhf	70,990	−	−	−	+	−	+
S11	Uninfected control	N/A	−	+	−	+	−	+
S12	Uninfected control	N/A	−	+	−	+	−	+

Blood copy numbers indicate the level of haemoplasma infection at the time of collecting saliva samples as determined by quantitative real-time PCR, according to the method reported by Tasker et al (2003a, b): + denotes a sample where haemoplasma DNA was detected by real-time PCR whilst − denotes that no haemoplasma DNA was detected by real-time PCR.

organisms within the salivary tissues and saliva. Interestingly, the two CMhm-infected cats that gave positive results for haemoplasma DNA in their salivary glands were those which had the highest copy numbers of CMhm in their blood (cats; G3 and G5; Table 2). Blood contamination of the saliva fractions or salivary gland samples in the CMhm-infected cats could have resulted in the positive haemoplasma results obtained for these samples. No gross evidence of blood contamination was apparent in the saliva fraction samples although this does not rule out microscopic blood contamination. The salivary gland samples, collected at necropsy, would inevitably have contained some blood. It is not possible to determine whether the positive PCR results obtained in these two cats were due to the detection of CMhm DNA within haemoplasma organisms attached to the surface of the erythrocytes in contaminating blood or due to the detection of CMhm DNA within organisms not attached to erythrocytes but free within the

Table 2. Salivary gland sampling results

Cat ID	Infecting haemoplasma species	Blood haemoplasma copy number (per μl of blood)	Salivary gland	
			Haemoplasma DNA	28S rDNA
G1	CMhm	184	−	+
G2	CMhm	702	−	+
G3	CMhm	44,716	+	+
G4	CMhm	702	−	+
G5	CMhm	122,186	+	+
G6	Mhf	196,818	−	+
G7	Mhf	19	−	+
G8	Mhf	353	−	+

Blood copy numbers indicate the level of haemoplasma infection at the time of collecting salivary gland samples as determined by quantitative real-time PCR, according to the method reported by Tasker et al (2003a, b): + denotes a sample where haemoplasma DNA was detected whilst − denotes samples where no haemoplasma DNA was detected.

saliva or salivary gland cells. If saliva was to act as a route of haemoplasma transmission, it may be that organisms can localise within salivary gland tissue without being associated directly with erythrocytes, as has been shown in the spleen (Maede and Murata 1978, Maede 1979). In situ hybridisation studies are required to elucidate the precise origin of the CMhm DNA in the salivary tissue.

28S rDNA was detected in all of the salivary gland samples and the majority of saliva samples, confirming the presence of amplifiable DNA in all of these samples. Thus a negative result on haemoplasma PCR on the same samples should not have arisen due to PCR inhibition and is likely to indicate either the absence of haemoplasma DNA in the sample or its presence at a level below the sensitivity limit of the PCR assay of 1.7 (Mhf) or 3.6 (CMhm) copies per PCR (Tasker et al 2003a, b).

However, three of the 12 saliva supernatant samples and two of the 12 cell pellet samples were negative for 28S rDNA by PCR. Although this may have been caused by the presence of PCR inhibitors, alternative explanations include the presence of only very low numbers of cells in the saliva, poor elution of those cells from the swab, or the presence of food debris within the mouth at the time of saliva collection which contributes to the subsequent cell pellet but contains no feline 28S rDNA. We cannot rule out that inhibition within those specific PCRs caused the negative 28S rDNA PCR results, but feel that a lack of cellular material is a likely contributing factor in view of the positive 28S rDNA PCR results obtained for the other samples and the positive control reaction.

This preliminary study was limited by the small number of cats available and the variability in haemoplasma blood copy numbers present at the time of sampling. However, the methodology was successful in demonstrating the use of quantitative real-time PCR to detect haemoplasma DNA in saliva and salivary gland tissue samples. To ensure that the presence of haemoplasma DNA within the saliva or salivary gland samples was not due to blood contamination, in situ hybridisation studies would be required to localise haemoplasmal DNA. Tissue has been archived for this purpose to expand on this work in the future. Experimental transmission studies will ultimately be the only method of confirming whether saliva acts a mode of transmission for CMhm infection.

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