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# Prevalence of feline haemotropic mycoplasmas in convenience samples of cats in Germany☆

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The aim of this prospective study was to evaluate the prevalence of feline haemotropic mycoplasmas in Germany, to determine probable risk factors for these infections and to compare the diagnostic value of microscopic examination of blood smears to polymerase chain reaction (PCR). For the prevalence study, convenience samples (Ethylene diamine-tetraacetic acid (EDTA) blood) from 262 (64.5% male and 35.5% female) cats were included. A PCR for the detection of *Mycoplasma haemofelis* (MHF) and '*Candidatus* Mycoplasma haemominutum' (CMH) as well as a feline leukaemia virus (FeLV)/feline immunodeficiency virus (FIV) enzyme-linked immunoassay was performed. Blood smears from 224 cats were examined and the sensitivity and specificity of the microscopic diagnosis were determined. The prevalence of CMH, MHF, and CMH/MHF co-infection was 22.5%, 4.5%, and 0.8%, respectively. CMH was significantly associated with male gender ($P = 0.047$), older age ($P = 0.0015$) and both FeLV ($P = 0.002$) and FIV infections ($P < 0.0001$). However, there was no association between the presence of anaemia and CMH/MHF infection. The respective sensitivity and specificity of the microscopic diagnosis were 10.3% and 87.1% for a CMH infection and 0.0% and 98.0% for MHF infection.

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Haemotropic mycoplasmas are extracellular, pleomorphic organisms which are attached to the surface of feline erythrocytes (Jain and Keeton 1973). Recent sequence analyses of the *16S rRNA* genes indicated that they belong to the genus *Mycoplasma* (Rikihisa et al 1997, Foley et al 1998, Neimark et al 2001, 2002). Following molecular characterisation of the *16S rRNA* gene, two feline haemotropic mycoplasmas were recognised (Foley et al 1998) as follows: *Mycoplasma haemofelis* (MHF) (Neimark et al 2002) and '*Candidatus* Mycoplasma haemominutum' (CMH) (Foley and Pedersen 2001). Recently, a third species of feline haemotropic mycoplasmas named '*Candidatus* Mycoplasma turicensis' (Willi et al 2006a,b) has been identified.

Until the development of polymerase chain reaction (PCR) assays for the detection of specific fragments of the organisms' nucleic acid sequence (Jensen et al 2001), the only test available was the microscopic examination of blood smears. However, due to low sensitivity and moderate to high specificity (Tasker et al 2003), the latter is not a reliable diagnostic test (Foley et al 1998, Jensen et al 2001, Westfall et al 2001, Tasker et al 2003).

Since the development of a PCR assay for the detection of feline haemotropic mycoplasmas, several studies have investigated the prevalence of infections with CMH and MHF in different countries including South Africa (Lobetti and Tasker 2004), the USA (Hackett et al 2006, Eberhardt et al 2006, Ishak et al 2007), Australia (Tasker et al 2004), Switzerland (Willi et al 2006b), the United Kingdom (Tasker et al 2003), and Japan (Watanabe et al 2003). To our knowledge, the prevalence for haemotropic mycoplasmas in non-selected samples has not yet been addressed in Germany.

Therefore, the aim of this study was to determine the prevalence of CMH and MHF

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infections in Germany using PCR technology as well as the evaluation of probable risk factors associated with feline haemotropic mycoplasma infection including the influence of gender, age and concurrent retroviral infections. The pathogenicity of CMH and MHF was assessed by investigating the association between infection and anaemia.

In a second part of the study, the use of the 'traditional' microscopic examination of blood smears for the diagnosis of either an MHF or CMH infection compared to the reference method, the detection of mycoplasma DNA by PCR, is evaluated.

## Material and methods

### *Prevalence study*

This study was performed prospectively and convenience samples were collected between May and August 2005. The term 'prevalence' when used in this manuscript refers to the number of cats testing positive for mycoplasma organisms in a convenience sample of cats. Ethylene diamine-tetraacetic acid (EDTA) blood samples from all cats presented to the Clinic for Small Animals, Justus-Liebig-University Giessen, Germany and consecutive blood samples submitted to the Vet Med Labor GmbH, Ludwigsburg, Germany were included. At the Clinic for Small animals, the majority of cats were presented due to internal medical problems (approximately 70%) whereas approximately 30% were shown due to surgical diseases or were sampled for a pre-anaesthetic check. Occasionally, specimens from healthy cats belonging to staff or blood donors at the clinic were submitted for a general heath check.

At Vet Med Labor GmbH, Ludwigsburg, samples from practitioners and veterinary clinics were received for the same reasons, however, in many cases the history of the patient was not known.

Inclusion criteria were available data on the gender of the cats and a sufficient sample volume (approximately 1.0 ml) for both haematological and PCR analyses. If available, the age of the cats was recorded. Samples taken from the same individual at follow-up examinations were excluded from the prevalence study.

Haematology was performed on EDTA whole blood samples with the multi-species haematology analyser ADVIA 120 either in the Clinic for Small Animals, Clinical Pathophysiology and Clinical Pathology or at the Vet Med Labor GmbH. Blood samples were centrifuged within 24 h of sampling and the majority of EDTA plasma was separated from the erythrocytes to determine the feline leukaemia virus (FeLV)/feline immunodeficiency virus (FIV) status. Therefore, concentrated erythrocytes and the remaining EDTA plasma (approximately 200 µl) were the basis for the detection of MHF and CMH infection with PCR whereas whole blood was used for samples submitted to Vet Med Labor. Analyses for FeLV antigen and FIV antibodies were performed at the Vet Med Labor GmbH with a commercial enzyme-linked immunoassay (ELISA) (Petcheck FeLV and Petcheck Plus Anti-FIV, Idexx Laboratory, Westbrook, USA) using a SLT Spectra microplate reader (Tecan Deutschland GmbH, Germany). The FeLV/FIV ELISAs were performed according to the manufacturer's instructions. If the sample volume was not sufficient for both analyses, priority was given to the FeLV ELISA.

### *DNA extraction and PCR amplification*

DNA was extracted and purified from 200 µl concentrated erythrocytes anticoagulated with EDTA (Clinic for Small Animals, Justus-Liebig-University Giessen) or 200 µl EDTA whole blood (Vet Med Lab GmbH) using the QIAamp DNA Blood Mini Kit (Quigen, Hilden, Germany), according to the manufacturer's instructions.

The PCR assay for DNA detection and differentiation of MHF and CMH was modified based on the method described previously (Jensen et al 2001).

To increase the analytical specificity of the PCR assay, restriction fragment length polymorphism (RFLP) analysis was performed on positive PCR amplicons: both MHF and CMH PCR products were digested with RsaI according to the recommendations of the manufacturer (NEB) leading to two 85 bp fragments for true positive MHF amplicons and to 108 and 85 bp fragments for true positive CMH PCR products, respectively. The PCR amplification products were identified by ethidium bromide fluorescence after electrophoresis in 2% agarose gels. For the detection of the RFLP products 2% metaphor agarose gels have been applied.

In each PCR, a known amount of plasmid DNA near the detection limit of the PCR (25 copies) derived from positive MHF and CMH blood samples were included as positive and sensitivity controls.

A blank control (no template control, NTC) as well as pure water treated as a patient sample was included as negative and extraction controls.

All DNA preparations were checked for the presence of inhibitory substances prior to PCR analysis by measurement of spiked extraction controls according to the quality standards for microbiological diagnostics of infectious diseases (MIQ) (Roth et al 2001). Samples with inhibitory substances were excluded.

### Assessment of sensitivity and specificity of microscopic diagnosis

Fresh specimens are required for the microscopic diagnosis and, therefore, only EDTA blood samples collected from cats presented in the Clinic for Small Animals, Justus-Liebig-University Giessen were included for sensitivity and specificity calculations.

In this part of the study, repeat samples from the same cat were not excluded. Blood smears were prepared within 4 h post sampling and again after 24 h in order to assess the effect of sample ageing on the microscopic detection of haemotropic mycoplasmas. A May Grünwald Giemsa stain was performed on batches of approximately 50 slides. Before staining, the staining solutions were freshly prepared and filtrated using a cellulose-containing filter paper. The microscopic analysis was performed by a board certified clinical pathologist (NB) blinded to the cats' clinical signs, laboratory findings and CMH/MHF PCR results. At least 10 high power fields with cells set in a monolayer were assessed at 1000-fold magnification. Only areas free from artefacts that may have interfered with the detection of haemotropic mycoplasmas, such as precipitated staining solution, were examined. The percentage of slides with artefacts precluding assessment was noted, and these specimens were excluded from the calculation for sensitivity, specificity, negative and positive predictive values of the microscopic examination.

### Statistical analysis

Based on the PCR assay results, the cats were defined as CMH and/or MHF infected. A haematocrit value less than 0.24 l/l was considered anaemic. The data obtained were analysed with the MedCalc (MedCalc version 9.0.1.1 for Windows© 1993–2006 Frank Schoonjans; http://www.medcalc.be/index.php) statistical software.

A Kolmogorov Smirnov test was applied to verify the assumption of normality. In case of normal distribution of data, a *t*-test was used and a Mann–Whitney test was applied as non-parametric test.

Frequency distributions for each PCR based category were calculated for cats with and without anaemia, for male and female cats, as well as for cats with and without underlying FeLV and FIV infections. Results were analysed with a Fisher's exact test. In addition, the odds ratio was used to determine the influence of gender or concurrent retroviral infections on the probability of an infection with haemotropic mycoplasmas.

Frequency distributions were calculated for the results of cytological detection of haemotropic mycoplasmas on blood smears prepared either within 4 h or 24 h after sampling. For all analyses, values of $P < 0.05$ were considered significant.

## Results

### Prevalence study

EDTA blood samples obtained from 262 (64.5% male and 35% female) cats presented at the Clinic for Small Animals, Justus-Liebig-University Giessen, Germany ($n = 171$) and specimens submitted to the Vet Med Labor GmbH, Ludwigsburg, Germany ($n = 91$) were included. Extraction and PCR controls were all negative. Inhibitory substances were not detected in any of the samples and all underwent PCR analysis. The PCR revealed a CMH infection in 61/262 cats including two cats with a CMH/MHF dual infection. A CMH mono infection was present in 59/262 cats consistent with a prevalence of 22.5%. Male cats were more likely to be CMH infected than female cats (Table 1). CMH was significantly associated with male gender and both FeLV and FIV infections (Table 1). The presence of anaemia and a CMH/MHF dual infection, however, had no significant effect (Table 1).

Fourteen of 262 cats (5.3%) were infected with MHF. In 12/262 (4.5%), an MHF single infection was present whereas a CMH/MHF co-infection was diagnosed in 2/262 cats (0.8%).

In contrast to CMH infection, no significant association between MHF infection and gender, FeLV/FIV status, and anaemia was detected.

None of the MHF and CMH/MHF co-infected cats was anaemic. There was no significant difference between the frequency of anaemia in MHF positive and negative cats (Table 1). The mean haematocrit value was equal in both

**Table 1**. Gender, FeLV/FIV infection status and presence of anaemia of cats with positive and negative PCR results for CMH and MHF ($n = 262$)

| Variable (number of cats) | CMH* | | | | | MHF* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of PCR negative cats (%) | Number of PCR positive cats (%) | Odds ratio | 95% Confidence interval | *P*-value | Number of PCR negative cats (%) | Number of PCR positive cats (%) | Odds ratio | 95% Confidence interval | *P*-value |
| *Gender (n = 262)* | | | | | | | | | | |
| Female | 78 (38.8) | 15 (24.6) | 1.00 | | | 87 (35.1) | 6 (42.9) | 1.00 | | |
| Male | 123 (61.2) | 46 (75.4) | 1.94 | 1.01−3.71 | 0.047 | 161 (64.9) | 8 (57.1) | 0.72 | 0.24−2.14 | 0.574 |
| *FeLV (n = 206)* | | | | | | | | | | |
| Negative | 163 (100.0) | 39 (90.7) | 1.00 | | | 193 (98.0) | 9 (100.0) | 1.00 | | |
| Positive | 0 (0.0) | 4 (9.3) | nd | nd | 0.002 | 4 (2.0) | 0 (0.0) | nd | nd | 1.000 |
| *FIV (n = 199)* | | | | | | | | | | |
| Negative | 156 (100.0) | 37 (86.0) | 1.00 | | | 184 (96.8) | 9 (100.0) | 1.00 | | |
| Positive | 0 (0.0) | 6 (14.0) | nd | nd | <0.0001 | 6 (3.2) | 0 (0.0) | nd | nd | 1.000 |
| *Anaemia (n = 262)* | | | | | | | | | | |
| No | 185 (92.0) | 58 (95.1) | 1.00 | | | 229 (92.3) | 14 (100.0) | 1.00 | | |

CMH = *Candidatus* Mycoplasma haemominutum; FeLV = feline leukaemia virus; FIV = feline immunodeficiency virus; MHF = *Mycoplasma haemofelis*; nd = Not done.

*Two male, non-anaemic CMH/MHF co-infected cats are included which are both testing negative for FeLV and FIV.

CMH infected ($0.37 \pm 0.01$ l/l; $n = 61/262$; $P = 0.626$) and non-infected cats ($0.37 \pm 0.01$ l/l; $n = 201/26$). MHF positive cats showed a higher mean haematocrit value ($0.42 \pm 0.07$ l/l; $n = 14/262$) than MHF negative cats ($0.37 \pm 0.08$ l/l; $n = 248/262$). However, this difference was not significant ($P = 0.626$).

In 247/262 (94.3%) cats, age data were available. Cats with positive MHF PCR result were older (median age 10.0 years; range 2.0–25.0 years; $n = 13/247$ cats with available data) than non-infected cats (median age 8.0 years; range 0.2–22.0 years; $n = 234/247$). However, the difference between the groups was not significant ($P = 0.245$). Cats tested positive for CMH were significantly older than CMH negative cats. The median age was 6.0 years (range 0.2–22.0 years; $n = 191/247$) in CMH negative cats while it was 10.0 years (range 2.0–25.0 years; $n = 56/247$; $P = 0.0015$) in cats with a positive PCR result for CMH.

### *Diagnostic sensitivity and specificity of blood smears*

Blood smears were prepared from 224 feline EDTA blood samples obtained from 171 cats. In 24/224 (10.7%) of the blood smears made within 4 h after sampling and 47/224 (21.0%) blood films prepared after 24 h could not be evaluated because large amounts of fine basophilic material consistent with precipitated staining solution. Of slides made within 4 h after sampling, 200/224 were of sufficient quality for microscopic examination. The respective sensitivity and specificity for the microscopic examination were 10.3% and 87.1% for detection of a CMH infection (3/200 true positive, 149/200 true negative, 22/200 false positive, and 26/200 false negative samples). Based on a prevalence of 22.5% for a CMH infection, positive and negative predictive values were 18.5% and 77.4%, respectively. Sensitivity and specificity were 0% and 98% for cytological detection of an MHF infection when compared to PCR results (0/200 true positive, 183/200 true negative, 6/200 false positive, and 11/200 false negative specimen). Given a prevalence of 4.5% for MHF, positive and negative predictive values were 0.0% and 95.4% for diagnosis of an MHF infection.

One-hundred-and-seventy-seven of 224 slides made 24 h after sampling were of sufficient quality for microscopic examination. Compared to the PCR gold standard, sensitivity and specificity of the microscopic examination were 37.5% and 58.2% for detection of a CMH infection (9/177 true positive, 89/177 true negative, 64/177 false positive, and 15/177 false negative samples). Positive and negative predictive values for the diagnosis of a CMH infection by microscopic evaluation of a blood smear were 20.3% and 76.6%, respectively. The sensitivity for detection of an MHF infection was 88.9% and the specificity was 61.9% for the detection of an MHF infection (8/177 true positive, 104/177 true negative, 64/177 false positive, and 1/177 false negative). Positive and negative predictive values were 9.9% and 99.2% for the diagnosis of an MHF infection.

Regarding the diagnosis of both CMH and MHF by cytology, the Fisher's exact test revealed a significant difference between cytological results obtained from smears prepared within 4 h after sample collection and those that were prepared after 24 h ($P < 0.0001$).

## Discussion

The prevalence of haemotropic mycoplasmas in Germany detailed here is comparable to data reported from Australia (Tasker et al 2004). A higher percentage of infected cats was demonstrated in South Africa (Lobetti and Tasker 2004) and Japan (Watanabe et al 2003), whereas, lower prevalence of CMH, MHF and CMH/MHF co-infection was reported from England (Tasker et al 2003) and Switzerland (Willi et al 2006b) as well as from northern Florida (Luria et al 2004), Arizona (Eberhardt et al 2006) and various other states in the USA (Hackett et al 2006).

In order to interpret the varying prevalence of haemotropic mycoplasmas in different countries the highly variable number of cats included in the studies must be taken into account (range 21–713 cats). Moreover, different inclusion criteria also influenced the results. In the majority of studies (Willi et al 2006b) including our own, both healthy and diseased cats were evaluated. However, other investigators assessed only healthy blood donors, a fact which may explain the comparatively low prevalence of haemotropic mycoplasmas reported in this study (Hackett et al 2006). In the Japanese prevalence investigations, only ill cats suspected of having a mycoplasmal infection were included which explains the relatively high prevalence of haemotropic mycoplasmas (Watanabe et al 2003). In the study by Eberhardt et al (2006) only samples from feral and relinquished cats with unknown

health status were evaluated. Regional differences may also have influenced the results. Willi et al (2006b) recently demonstrated a significantly higher prevalence of haemotropic mycoplasmas in the South/West of Switzerland than in the rest of the country.

It must be considered that the study was performed using a convenience sample population, the limitations of which have been discussed in previous reports (Sukura et al 1992). Therefore, no conclusions can be drawn regarding the general prevalence of CMH and MHF in Germany.

In the cats evaluated in the present study, an infection with haemotropic mycoplasmas was not associated with anaemia or lower haematocrit values compared to the non-infected group. Though this finding is in agreement with another recent study (Willi et al 2006b), it contradicts data from previous investigators who demonstrated a significant inverse correlation between the amount of MHF DNA present in the blood and the haematocrit value as well as a higher probability of cats with lower haematocrit values to be infected with MHF (Tasker et al 2004, Lobetti and Tasker 2004).

A probable reason for this discrepancy between different studies can be explained by the fact that the stage of infection is not known in cats with a positive PCR result. As cats do not reliably eliminate mycoplasma organisms (Foley et al 1998, Berent et al 1998, Westfall et al 2001), MHF positive cats may be carriers for this organism and could have demonstrated clinical signs indicative of MHF infection at the time of initial infection.

Due to the small number of anaemic cats, associations between the infection status and the presence of anaemia might have been missed.

Infection with either FeLV or FIV has been found to be associated with an increased risk for co-infection with MHF or CMH (Luria et al 2004). Our results clearly indicated that older, male, FeLV and FIV infected cats were more likely to be infected with CMH, however, this contradicts a recent study which fails to demonstrate an association between retrovirus and haemoplasma infections (Willi et al 2006a). Risk factors for an infection with MHF were not identified in our investigations.

While it is known that haemotropic mycoplasmas may detach from erythrocytes to the plasma within 2 h after sample taking (Alleman et al 1999), a limitation of our study was the delay in plasma removal from the samples. Although this may have an impact on the detection of organisms by PCR, partial removal of plasma was necessary to perform FeLV/FIV tests for the assessment of the association between retrovirus and haemoplasma infections. However, approximately 200 µl of plasma were left with the pelleted erythrocytes for PCR analysis, so that the detection of detached organisms was still possible.

The low sensitivity for a cytological diagnosis of CMH and MHF of 10.3% and 0.0% is comparable to a previous study reporting a sensitivity of 11.1% including equivocal blood smears for the diagnosis of mycoplasma infection (Tasker et al 2003). Generally, cytology is known to be problematic for diagnosing a mycoplasma infection (Foley et al 1998, Jensen et al 2001, Westfall et al 2001). The specificity for a microscopical diagnosis of CMH and MHF was 87.1% and 98.0% in the samples investigated here which is similar to a previously reported specificity of 84% (Tasker et al 2003). In the previous report, however, the mycoplasma strains were not differentiated.

Storage of blood samples for 24 h resulted in an increase in sensitivity along with a decrease in specificity for the cytological diagnosis of either CMH or MHF infection. This may be caused by the increased amount of precipitated material observed after 24 h which is easily mistaken for haemotropic mycoplasmas. No explanation can be given for the increase of precipitated material after 24 h as samples of fresh blood and blood stored for 24 h were always stained in batches.

Haemotropic mycoplasmas may detach from the erythrocyte membrane during the storage in EDTA (Alleman et al 1999) and, therefore, organisms dislodged from erythrocytes may be mistaken as precipitated material. On the other hand this material was also present in PCR negative samples and, therefore, contributes to an increase of the rate of false positive diagnoses which explains the decreased specificity of cytological diagnosis after 24 h.

Our results clearly demonstrated that microscopic diagnosis of feline haemotropic mycoplasmas cannot be recommended as a screening tool: a finding which is in agreement with previous studies (Foley et al 1998, Jensen et al 2001, Westfall et al 2001, Tasker et al 2003).

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