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Original article

In vivo transmission studies of '*Candidatus* Mycoplasma turicensis' in the domestic cat

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Abstract – The natural transmission routes of the three feline haemotropic mycoplasmas – *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum', and '*Candidatus* Mycoplasma turicensis' (CMt) – are largely unknown. Since CMt has been detected in the saliva of infected cats using PCR, we hypothesised that direct transmission via social or aggressive contact may occur. The aim of this study was to evaluate this transmission route. CMt-positive saliva and blood samples were obtained from three prednisolone-treated specific pathogen-free (SPF) cats that were infected intraperitoneally with CMt. Five SPF cats were inoculated with CMt-positive saliva or blood subcutaneously to mimic cat bites, and five cats were inoculated orally with blood or oronasally with saliva to mimic social contact. Blood samples were monitored for CMt infection using quantitative real-time PCR and for seroconversion using a novel western blot assay. Neither oronasal nor subcutaneous inoculation with CMt-positive saliva led to CMt infection in the recipient cats, as determined by PCR, independent of prior prednisolone treatment. However, when blood containing the same CMt dose was given subcutaneously, 4 of the 5 cats became PCR-positive, while none of the 5 cats inoculated orally with up to 500 µL of CMt-positive blood became PCR-positive. Subsequently, the latter cats were successfully subcutaneously infected with blood. All 13 CMt-exposed cats seroconverted. In conclusion, CMt transmission by social contact seems less likely than transmission by aggressive interaction. The latter transmission may occur if the recipient cat is exposed to blood from an infected cat.

haemotropic mycoplasma / transmission / '*Candidatus* Mycoplasma turicensis' / real-time TaqMan PCR / seroconversion

1. INTRODUCTION

Three haemotropic mycoplasmas, the causative agents of feline infectious anaemia, have been described in cats: *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt) [6, 23, 25, 27, 36]. Recently, a fourth species similar to '*Candidatus* M. haematoparvum' has been suggested to infect

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cats [29]. The pathogenic potential of the feline haemotropic mycoplasma isolates studied to date varies, and immunosuppression or pre-existing retroviral infections may potentiate the severity of the anaemia [9, 36].

The diagnosis of feline haemotropic mycoplasmas (a.k.a. haemoplasmas) has been limited because microscopic detection of the agents in blood smears is unreliable [32, 37]. Recently, sensitive PCR assays have been introduced and are now considered to be the gold standard for the detection and differentiation of all feline haemoplasma species [33, 36, 37]. Using PCR, the worldwide distribution of feline haemotropic mycoplasma infections has been documented [7, 20, 29, 32, 34, 41]. To date, no commercial routine serologic assay is available, and serodiagnostics have depended on crude Mhf antigens [1, 5]. Recently, an immunodominant protein of *Mycoplasma suis,* a porcine haemoplasma species, was identified as HspA1, a surface-localised dnaK analogue [15–17].

The natural mode of feline haemoplasma transmission remains controversial. Experimental transmission by the intraperitoneal, intravenous, and oral inoculation of infected blood has been successful [4]. However, these early studies were performed prior to the introduction of specific molecular assays, so it is not known which feline haemoplasma species were studied. More recently, blood transfusions have been reported to be a source of Mhf and CMhm infections [8, 37]. Furthermore, blood-sucking arthropods may be involved in the transmission of feline haemoplasmas. While almost 2 000 unfed *Ixodes* spp. ticks collected from vegetation in Switzerland tested PCR-negative for feline haemoplasmas, and positive results for CMhm and CMt were only obtained from some *Ixodes* spp. and *Rhipicephalus* spp. ticks collected directly from animals [39], CMhm DNA has been detected in unfed *Ixodes ovatus* ticks in Japan [31]. This suggests a transstadial transmission of CMhm at least in the latter tick species. Moreover, Mhf and CMhm DNA was detected in cat fleas (*Ctenocephalides felis*) collected from experimentally and naturally infected cats and in flea faeces [18, 28, 39, 42, 43], but the transmission of Mhf and CMhm via *C. felis* has not been proven [42, 43]. Recently, an investigation of shedding patterns by real-time PCR led to the detection of CMt DNA in saliva and faeces up to nine weeks after experimental CMt infection [39]. In the same study, however, Mhf and CMhm DNA were not detected in the saliva and faeces of some naturally infected cats, despite high blood haemotropic mycoplasma loads [39] and most recently CMhm but not Mhf was detected in the saliva and salivary glands of experimentally infected cats [2]. We thus hypothesised that feline haemoplasmas, and particularly CMt or CMhm, could be transmitted by direct contact (social or aggressive) via saliva [39, 40]. Our hypothesis is supported by the general increase of feline haemoplasma infections, and particularly Mhf, CMhm and CMt infections, in male cats [21, 32, 37, 38]. Moreover, an early study reported that a history of cat bite-associated abscesses increased the relative risk of infection with haemotropic mycoplasmas [11]; however, the particular haemoplasma species could not be molecularly differentiated at the time of that study.

The aim of the present study was to address this hypothesis and to investigate the possibility of direct transmission of CMt between cats. To mimic aggressive contact and cat bites, saliva or blood samples from CMt-infected cats were inoculated subcutaneously into recipient cats, while social contact between cats was simulated by oronasal and oral inoculation. All cats were monitored following CMt exposure, and blood samples were assessed for CMt infection using sensitive real-time PCR and a novel western blot assay.

2. MATERIALS AND METHODS

2.1. Cats

Thirteen specific pathogen-free (SPF) cats (Liberty Research, Waverly, NY, USA) were included in this study. They were kept in a confined university facility under hygienically ideal conditions. All experiments were officially approved by the veterinary office of the canton of Zurich (101/2007). The cats were kept in groups in large rooms furnished with running boards, climbing trees, ladders, hammocks, elevated sleeping places, and shielding retreat

areas, under optimal ethological conditions. Prior to the start of the experiment, each cat was clinically examined, and blood and serum samples and conjunctival, oropharyngeal, and rectal swabs were collected to verify their SPF status. The cats were found to be negative when the samples were tested by real-time TaqMan PCR or RT-PCR, as described [12–14, 19, 22, 24, 30, 35–37], as follows. The blood samples were tested for CMt; Mhf; CMhm; feline parvovirus, calicivirus, and coronavirus; *Bartonella henselae*; and feline leukaemia and immunodeficiency virus provirus. The conjunctival swabs were tested for feline calicivirus, feline herpesvirus-1, and for *Chlamydophila felis.* The oropharyngeal swabs were tested for feline calicivirus and feline herpesvirus-1, and the rectal swabs were tested for feline coronavirus and feline parvovirus. In addition, the serum samples tested negative for antibodies to feline coronavirus [26]. Cats were blood-typed using a commercial gel column technique (ID-Gel Test Feline A+B Typing; DiaMed AG, Cressier sur Morat, Switzerland): 11 cats were of blood type A and two cats were of blood type B. The two latter cats were siblings. Three castrated adult male cats (2 and 3 years of age) were infected intraperitoneally with CMt to obtain infectious saliva and blood samples (amplificatory cats; Cats X, Y, and Z). Ten juvenile cats (two months of age) housed in two groups of 5 cats each (groups A and B) were used as recipient cats for the transmission studies. Following the conclusion of this study, the three amplificatory cats were adopted; the ten recipient cats continued to remain as study cats.

2.2. Clinical examination and sample and inocula collection

The clinical examinations were performed by three veterinarians. The complete haemograms of the amplificatory cats were performed prior to prednisolone treatment and prior to CMt infection, daily during saliva collection, and sporadically until 24 weeks p.i. for Cats X and Y and until 17 weeks p.i. for Cat Z. Saliva swabs (Primella, Migros Genossenschafts-Bund, Zurich, Switzerland) were collected every day once the blood of the cats was blood PCR-positive. To collect large volumes of saliva (> 1 mL) for the transmission study, saliva production was stimulated in Cats X and Y by the subcutaneous injection of neostigmine (Valeant Pharmaceuticals, Birsfelden, Switzerland; up to 0.05 mg/kg) 10 min prior to ketamine/midazolam narcosis (ketamine 20 mg/kg and midazolam 0.1 mg/kg, intramuscularly; Vétoquinol, Ittigen, Switzerland/Roche, Reinach, Switzerland). The saliva samples were divided into aliquots for inoculation and PCR. The samples for inoculation were kept at room temperature and administered within 20 min of collection; the samples for PCR were stored at –20 °C until use. EDTA-anticoagulated blood samples and serum were collected for PCR analysis, haematology, and western blot from the ten recipient cats prior to, and regularly after, the CMt inoculation. Body temperature and body weight were recorded at each sampling date.

2.3. Haematology

Haemograms were performed using a Cell-Dyn 3500 (Abbott, Baar, Switzerland). Packed cell volume (PCV) values between 33% and 45% (5% and 95% quantiles, reference range of the Clinical Laboratory, defined by identical methods from 58 healthy cats) were considered to be within the normal range; anaemia was defined as a PCV value below 33%. Modified Wright-stained blood smears (AMES Hema Tek slide stainer, Bayer, Zurich, Switzerland) and Diff-Quick-stained (Medion Diagnostics, Dudingen, Switzerland) smears were evaluated by light microscopy for CMt.

2.4. Intraperitoneal infection

The three amplificatory cats were infected by the intraperitoneal administration of CMt, as previously described [4]. Cats X, Y, and Z received 9×10^5 copies of CMt in 0.9–1 mL of infectious, heparinised blood that had been preserved in 20% DMSO (Sigma-Aldrich, Buchs, Switzerland) and stored at –80 °C. The CMt used was derived from the CMt isolate previously described in "Cat 1" (passage 1) [36]. It had been passaged through an additional SPF cat before it was administered to Cats X and Y. Thus, it had been passaged twice prior to inoculation into Cats X and Y. For Cat Z, the original isolate had been passaged three times prior to inoculation. All three cats were administered two doses of methylprednisolone acetate intramuscularly (10 mg/kg, Depo-Medrol, Pfizer AG, Zurich, Switzerland) in an attempt to immunocompromise the cats; Cats X and Y were injected 16 and 9 days prior to CMt inoculation, Cat Z was injected 8 and 1 days prior to CMt inoculation. Additionally, beginning at 14 days p.i., Cat Z received prednisolone orally (4 mg/kg/d, Streuli Pharma AG, Uznach, Switzerland) for 20 days.

During the peak bacteraemia of Cats X, Y, and Z, 40 mL of blood and 2 mL of bone marrow were collected. Bone marrow aspiration was performed on the

proximal humerus using Rosenthal type bone marrow aspiration needles (Monoject, 16 gauge × 2", Kendall, Mansfield, MA, USA) under short duration general anaesthesia and analgesia. The cats were administered acepromazine (Arovet AG, Zollikon, Switzerland, 0.01 mg/kg) and buprenorphine (Essex Chemie, Switzerland, 0.01 mg/kg) intramuscularly, followed by an intravenous injection of propofol (Fresenius, Bad Homburg, Germany; induction 5 mg/kg; maintenance 1 mg/kg every 2 min). Subsequently, the three amplificatory cats were administered antibiotic treatment with marbofloxacin (Vétoquinol) and/or doxycycline (Grünenthal GmbH, Mitlödi, Switzerland), as indicated in Figure 1.

2.5. Oronasal and subcutaneous exposure to CMt DNA-containing saliva

As a model for the direct transmission of CMt via saliva, to simulate aggressive and social contact, the saliva from the CMt-positive amplificatory cats was inoculated oronasally or subcutaneously into the recipient cats within 20 min of collection. Five recipient cats (group A) were exposed oronasally to the saliva (Tab. I, A I): one drop of saliva was administered to each nostril of the cats using a syringe, and the remaining saliva was administered orally. Each cat received a cumulative dose of 2.1 mL saliva containing 8×10^2 copies of CMt over a period of 19 days; inoculations took place on 17 of the 19 days. The samples were administered based on the available volume of saliva collected from the amplificatory cats; the volume of the PCR-positive transmitted samples ranged from 9–200 µL per recipient cat and day. An aliquot of 100 µL saliva containing 1×10^2 copies of CMt was inoculated subcutaneously into each of the five recipient cats in group B (Tab. I, B I). When inoculations A I and B I were unsuccessful, the cats were administered prednisolone in an attempt to immunocompromise the cats prior to and during the next CMt inoculation, as indicated in Table I (A II and B II). The development of saliva-associated abscesses necessitated the termination of prednisolone administration in group B, and cat 7 required treatment with amoxicillin-clavulanate (Synulox, 25 mg/kg, every 12 h for 9 days, Pfizer AG).

2.6. Oral and subcutaneous exposure to CMt-containing blood

Since the saliva inoculations, with or without prednisolone administration, did not lead to CMt

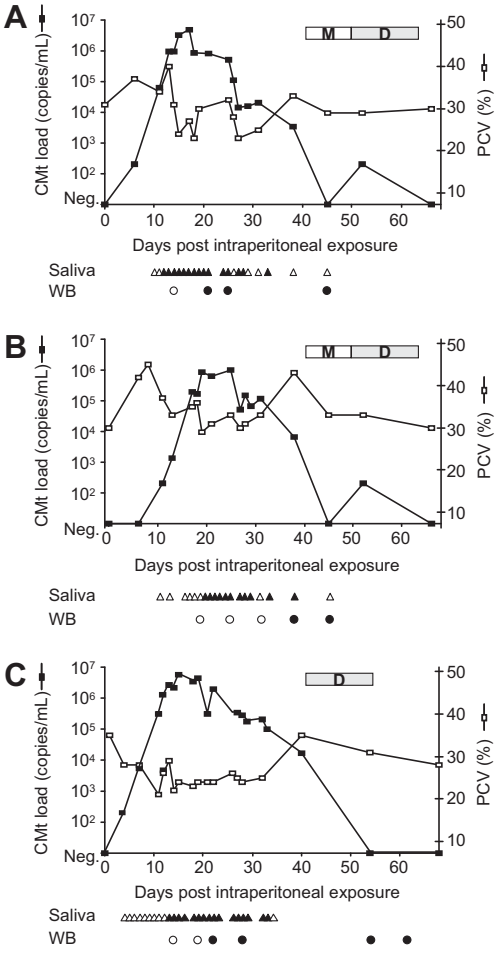

Figure 1. Intraperitoneal CMt infection of the three amplificatory SPF cats, Cats X (A), Y (B), and Z (C). Kinetics of CMt blood load (left *y*-axis, black squares), PCV value (right *y*-axis, open squares), shedding in saliva (triangles beneath the *x*-axis), and serology results as determined by western blot (WB; circles beneath the *x*-axis) throughout the course of CMt infection. The blood loads are presented as the log copy number of DNA template per mL of blood. PCV is presented as a percentage. The saliva samples were analysed by real-time PCR: PCR-positive swabs are indicated by black triangles, negative swabs are indicated by open triangles. Western blot-positive and -negative serum samples are indicated by black and open circles, respectively. From day 41 to day 50 p.i., Cats X and Y were administered marbofloxacin at 2 mg/kg every 24 h (white boxes marked "M"). Treatment was then switched to doxycycline at 10 mg/kg/d for 14 days (gray boxes marked "D"). Cat Z was administered only

Table I. Experimental setup of the CMt transmission studies: groups and numbers of cats, CMt exposure, immunosuppression protocol, inoculum (CMt-positive saliva or blood), inoculation route, and monitoring period.

Group of cats	CMt exposure	Administration of prednisolone	Number of cats	Inoculum (total copies of CMt)	Inoculation route	Monitoring period (weeks)
Group A	I. Transmission by saliva		5	2.1 mL saliva (8×10^2 copies)	Oronasal	4
	II. Transmission by saliva, immunosuppression	5 mg/kg/d for 8 days; 2.5 mg/kg/d for 29 days	5[a]	6.1 mL saliva (7×10^3 copies)	Oronasal	6
	III. Transmission by blood		5[a]	63 µL blood (8×10^3 copies)	Oral	10
	IV. Transmission by blood		5[a]	500 µL blood (4×10^5 copies)	Oral	9
	V. Transmission by blood		5[a]	50 µL blood (6×10^3 copies)	Subcutaneous	23
Group B	I. Transmission by saliva		5	100 µL saliva (1×10^2 copies)	Subcutaneous	6
	II. Transmission by saliva, immunosuppression	5 mg/kg/d for 8 days; 2.5 mg/kg/d for 14 days	5[b]	2 × 200 µL saliva (1×10^3 copies)	Subcutaneous	4
	III. Transmission by blood		5[b]	10 µL blood (1×10^3 copies)	Subcutaneous	30
	IV. Transmission by blood		1[b] (Cat 7)	10 µL blood (1×10^3 copies)	Subcutaneous	9

[a] Same cats used in experiment A I (Cats 1, 2, 3, 4, 5).
[b] Same cats used in experiment B I (Cats 6, 7, 8, 9, 10).

infection in the recipient cats, as determined by real-time PCR, by week 4–6 after the last saliva administration, the cats in both groups A and B were inoculated with CMt-positive blood (Tab. I, A III and B III). Each cat in group A was administered 63 µL of blood containing 8×10^3 copies of CMt (Tab. I, A III). The cats were offered the blood in a syringe, and they voluntarily licked and ingested the blood; no rejection of the blood was observed. When the cats remained PCR-negative at week 10, they were inoculated with a larger blood volume containing 4×10^5 CMt copies (Tab. I, A IV). When the cats again remained PCR-negative at week 9 following the larger dose, they were challenged subcutaneously with 6×10^3 CMt copies in 50 µL of CMt-positive blood (Tab. I, A V) in order to confirm that the cats could be infected with CMt. Group B cats received 1×10^3 CMt copies in 10 µL of blood subcutaneously (Tab. I, B III). In cat 7, which remained PCR-negative, the inoculation was repeated 14 weeks after the first subcutaneous inoculation (Tab. I, B IV).

2.7. TNA extraction

Total nucleic acids (TNA) were extracted from 100 µL of EDTA-anticoagulated blood and bone marrow using the MagNa Pure LC Total Nucleic Acid Isolation Kit I (Roche Diagnostics). The samples were stored at –20 °C prior to extraction. The TNA was eluted in 100 µL of elution buffer. Negative extraction controls consisting of 200 µL of phosphate buffered saline were prepared with each batch. The saliva samples and swabs in PBS were incubated at 40 °C for 10 min before TNA extraction, as previously described [10].

2.8. Quantitative real-time PCR

All TNA samples were analysed and quantified by real-time TaqMan PCR for the presence of CMt DNA, as previously described [36]. Water was used as a negative PCR control.

2.9. Serology

The recombinant HspA1 protein of Mhf (FJ463263) was used as an antigen. The 899 bp fragment was ligated into the pQE-30 Xa Vector (Qiagen, Hombrechtikon, Switzerland), which was then transformed into Top10 *Escherichia coli* cells (Invitrogen, Basel, Switzerland). Protein production, SDS-PAGE, and immunoblotting were performed as described previously [15], with the following modifications: protein expression was induced using 1 mM IPTG, and *E. coli* cultures were grown in LB medium containing 100 µg/mL of ampicillin. The 6xHis-tagged protein was purified by nickel affinity chromatography (Qiagen) or chelating sepharose (GE Health care biosciences, Uppsala, Sweden). A total of 10 µg of antigen was used per stripe, and the feline samples were incubated at a dilution of 1:100. Immunoreactive proteins were visualised using horseradish peroxidase-conjugated goat anti-cat IgG (Sigma-Aldrich) and 4-chloro-1-naphthol as substrate.

The western blot was evaluated with samples from three SPF cats infected with Mhf (6 weeks p.i.), CMhm (21 weeks p.i.), or CMt (8 weeks p.i.), in addition to samples from five haemoplasma-free SPF cats. To study seroconversion following CMt infection, the three amplificatory and ten recipient cats were tested prior to and at various time-points following CMt exposure.

2.10. Statistics

Statistical analyses were performed using the Excel add-in Analyse-it (Analyse-it Software, Leeds, UK) and Graph-Pad Prism (GraphPad Software, San Diego, CA, USA). For the correlation analyses, the Spearman rank correlation test was used (p_S). Continuous variables were analysed for significant differences among the three groups using the Kruskal-Wallis test (p_{KW}) and Dunn multiple comparisons post-test. The comparisons between two groups were made using the Mann-Whitney U-Test (p_{MWU}). A p-value less than 0.05 was considered significant.

3. RESULTS

3.1. Characteristics of cats infected intraperitoneally with CMt

CMt DNA was detected in blood samples, salivary swabs, and bone marrow collected from all three amplificatory cats (Fig. 1). All of the TNA extraction controls and negative PCR controls were PCR-negative. Cats X and Z became blood PCR-positive at the first sampling date, days 6 and 4 p.i., respectively; Cat Y became blood PCR-positive on day 11 p.i. (Fig. 1). The peak CMt blood load reached 10^6 copies/mL, while the bone marrow load reached 10^4 copies/mL. The cats remained blood PCR-positive until treatment (see below). Cats X, Y, and Z became anaemic, with a minimum PCV of 23%, 29%, and 21% at days 18, 19, and 11 p.i., respectively (Figs. 1A, 1B, and 1C). When the combined data from the three cats were analysed, the haemoplasma load was inversely correlated with the PCV value ($r = -0.35$; $p_S = 0.0057$), erythrocyte count ($r = -0.45$; $p_S = 0.0003$), and haemoglobin value ($r = -0.35$; $p_S = 0.0060$). A mild loss of appetite and apathy were observed in all three cats 2–3 weeks after infection.

3.2. CMt DNA shedding via saliva

Positive PCR results were obtained from the salivary swabs collected from all three amplificatory cats during the peak bacteraemia (Fig. 1). The CMt load in PCR-positive saliva samples ranged from 1×10^2 to 6×10^3 copies/mL of saliva (median: 7×10^2 copies/mL).

3.3. Transmission experiment: oronasal and subcutaneous exposure to CMt DNA-containing saliva

No evidence of CMt infection was detected in any of the recipient cats after attempted transmission of CMt via saliva, as determined by real-time PCR. The results were independent of prior prednisolone administration in the ten cats (Tab. II, A I and A II and B I and B II).

3.4. Transmission experiment: oral and subcutaneous exposure to CMt-containing blood

Neither low dose (8×10^3 CMt copies) nor high dose (4×10^5 CMt copies) oral exposure to CMt-positive blood led to infection of the five recipient cats in group A (Tabs. I and II, A III and A IV). These cats were subsequently

Table II. Outcome of CMt transmission studies (blood real-time PCR and serology results) in the groups and experiments described in Table I.

Group/CMt exposure	Cat 1		Cat 2		Cat 3		Cat 4		Cat 5	
	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology
A I.	–	–	–	–	–	–	–	–	–	–
A II.	–	–	–	+	–	–	–	–	–	–
A III.	–	–	–	+	–	–	–	–	–	–
A IV.	–	–	–	+	–	–	–	–	–	–
A V.	+	+	+	+	+	+	+	+	+	+
	Cat 6		Cat 7		Cat 8		Cat 9		Cat 10	
	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology
B I.	–	–	–	–	–	–	–	–	–	–
B II.	–	–	–	–	–	–	–	–	–	–
B III.	+	+	–	–	+	+	+	+	+	+
B IV.	nt	nt	–	+	nt	nt	nt	nt	nt	nt

nt: not tested.

inoculated subcutaneously with CMt-positive blood, and 14–45 days p.i. all cats in group A became PCR-positive and developed a peak blood CMt load of 10^3–10^5 copies/mL (Tab. II, A V; Figs. 2A–2E).

In group B, 4 of the 5 recipient cats subcutaneously inoculated with CMt-positive blood (Tab. I, B III) became PCR-positive after an average of 22 days (range: 19–33 days), with a peak blood load ranging from 10^3–10^5 copies/mL (Tab. II, Figs. 2F–2K). One cat (Cat 7) was administered antibiotic treatment for a saliva-associated abscess 22–30 days prior to blood inoculation and remained PCR-negative throughout the study. Cat 7 was one of the two cats with blood type B.

Clinical signs of infection (e.g. fever and weight-loss) were absent in the ten recipient cats. Anaemia was only observed in one case (Cat 1, group A) in which the PCV dropped from 37% to 28%, coinciding with peak CMt load of 9×10^5 copies/mL (Fig. 2A). Similarly, the peak bacteraemia in Cats 3 and 10 was accompanied by a slight decrease (by 7%) in the PCV (group A, Figs. 2C and 2K).

3.5. Comparison of experimental infections

A significant difference in maximum CMt blood load was observed between the amplificatory cats, the cats in group A, and the cats in group B ($p_{KW} = 0.0436$). The intraperitoneally infected cats showed a significantly higher bacterial load when compared to the subcutaneously exposed cats (groups A V and B III; $p_{MWU} = 0.0091$). A significant difference was also found in the length of time between CMt exposure and the first positive PCR result between the three groups ($p_{KW} = 0.0229$), and cats infected intraperitoneally became PCR-positive earlier after CMt exposure than cats infected subcutaneously ($p_{MWU} = 0.0091$). Additionally, cats infected intraperitoneally displayed a lower minimum PCV value than cats exposed subcutaneously ($p_{MWU} = 0.0182$).

3.6. Correlation of anaemia with CMt load

When all CMt PCR-positive cats ($n = 12$) were included in the analyses, the minimum PCV value was significantly negatively correlated with the maximum CMt blood load ($r = -0.84$; $p_S = 0.0006$; Fig. 3A). Moreover, the minimum PCV was significantly positively correlated with the length of time between CMt inoculation and the first positive PCR blood result ($r = 0.80$; $p_S = 0.0017$; Fig. 3B), and the peak CMt blood load was significantly negatively correlated with the length of time between CMt inoculation and the first positive PCR result ($r = -0.86$; $p_S = 0.0003$; Fig. 3C).

3.7. Antibiotic treatment

After doxycycline treatment, all three cats (X, Y, and Z) became persistently blood PCR-negative through the end of the study (17–24 weeks p.i.). The nine PCR-positive recipient cats were not administered any antibiotics, but they all became PCR-negative within 10–21 weeks p.i. and remained negative until the end of the observation period (7–10 weeks later).

3.8. Serology

The Mhf HspA1 fragment was recognised by sera from cats infected with Mhf, CMhm, or CMt, while sera from five haemoplasma-free SPF cats did not react against this antigen (Fig. 4A). All cats used in the CMt transmission study were seronegative prior to CMt exposure (Fig. 4B). After CMt challenge, all 13 cats became seropositive based on the western blot analysis (Fig. 4C). Weekly serum samples demonstrated seroconversion in the majority of these cats between 3 and 4 weeks p.i. (Figs. 1 and 2). Cats infected intraperitoneally with CMt demonstrated seroconversion between 3 and 6 weeks p.i. (Fig. 1) and remained seropositive until 13 to 15 weeks p.i. Thereafter, the bands observed in the western blot decreased in intensity and/or vanished over time. In group A, 4 out of the 5 cats seroconverted 3 to 4 weeks after exposure A V (Fig. 2A and 2C–2E), while one cat, Cat 2 (Fig. 2B), had already seroconverted 7 weeks after oronasal saliva inoculation (A II). Cat 2 was 1 of the 2 cats with blood type B. In group B, the four PCR-positive cats also seroconverted following

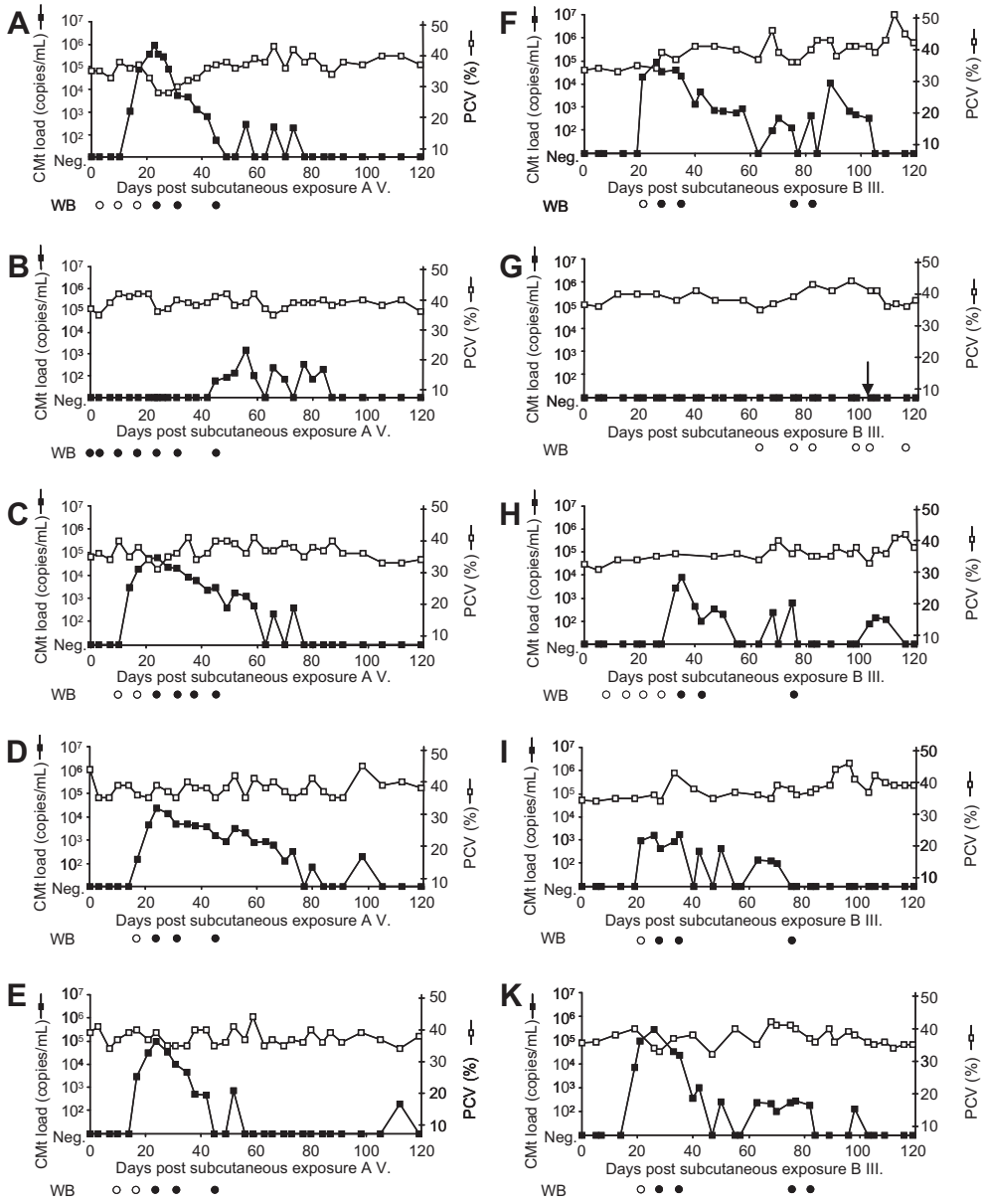

Figure 2. Results of transmission studies in ten SPF recipient cats after subcutaneous CMt exposures A V (group A; cats 1–5; A–E) and B III (group B; cats 6–10; F–K). The kinetics of CMt blood load (left *y*-axis, black squares), PCV value (right *y*-axis, open squares), and serology as determined by western blot (WB; circles beneath the *x*-axis) throughout the course of CMt infection. The blood load is presented as the log copy number of DNA template per mL of blood. The PCV is provided as a percentage. Western blot-positive and -negative serum samples are indicated by black and open circles, respectively. Cat 7 (B) received a second subcutaneous inoculation of CMt (Tab. I, exposure B IV) at week 14 p.i. (day 103 p.i.), as indicated by the black arrow.

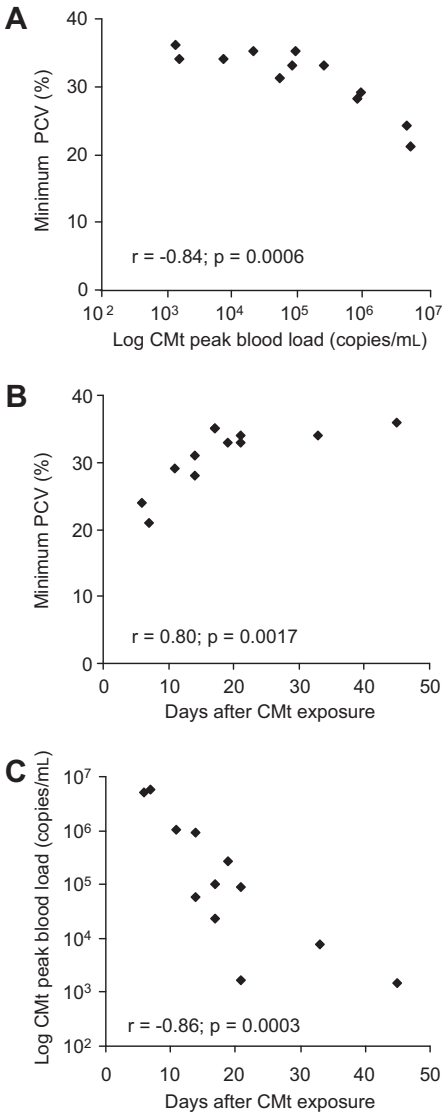

Figure 3. Correlation of anaemia and CMt load. The minimum PCV value was negatively correlated with the peak CMt blood load (A) and significantly positively correlated with the length of time between CMt exposure and the first positive PCR result (B). The peak CMt load was significantly negatively correlated with the length of time between CMt exposure and the first positive PCR result (C). The correlations were determined using the Spearman rank correlation test (*r* and *p* values indicated in the Figure).

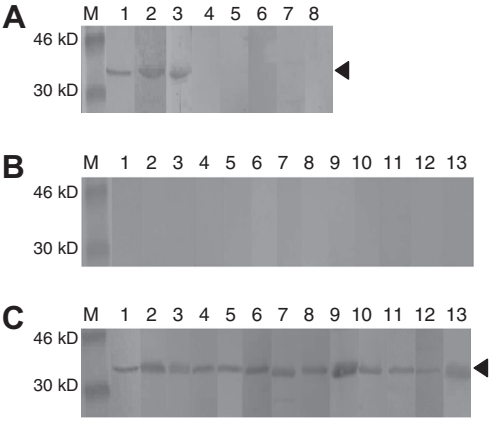

Figure 4. Western blot analyses of 16 experimentally haemoplasma infected cats and five SPF cats using a recombinant Mhf HspA1 as an antigen. (A) Western blot analysis of animals infected with different haemotropic Mycoplasmas (1: cat infected with Mhf; 2: cat infected with CMhm; 3: cat infected with CMt; 4–8: SPF cats). (B) Serum of groups A and B and the amplificatory cats collected prior to exposure. (C) Serum of groups A and B and the amplificatory cats collected post-exposure (5–11 weeks after inoculation with CMt-positive blood). M: Marker; 1–5: group A cats; 6–10: group B cats; 11–13: cats X, Y, and Z. Seroconversion occurred as early as three weeks p.i.; one cat seroconverted only at eight weeks after CMt inoculation. The arrow indicates the specific band (calculated size of 33.6 kDa).

CMt exposure (Tab. II, B III) between 3 and 5 weeks p.i. (Figs. 2F, 2H, 2I, and 2K). One cat, Cat 7 (Fig. 2G), remained PCR-negative throughout the study and subsequently seroconverted 8 weeks after a second subcutaneous inoculation of 1×10^3 CMt copies (Tab. II, B IV). All recipient cats remained seropositive until the end of the study.

3.9. Blood smears

None of the infected cats, regardless of the CMt blood load, showed characteristic haemoplasma-like inclusions that could be clearly identified by light microscopy of stained blood smears.

4. DISCUSSION

This was the first experimental study to investigate the direct transmission of CMt (e.g. transmission via social or aggressive contact between cats) using sensitive and specific real-time PCR assays. None of the cats oronasally or orally exposed to saliva or blood from CMt-infected cats became blood PCR-positive. The same was true following the subcutaneous inoculation of saliva. In contrast, subcutaneous exposure to infectious blood resulted in infection.

In a previous study, the shedding of CMt DNA via saliva was demonstrated in two experimentally infected cats during the early, but not at the late, phase of infection [39]. In the present study, CMt DNA in saliva was found in all three monitored cats. Although the CMt used in the present study had been further passaged in vivo, no differences were observed in the course and kinetics of the infection or the shedding pattern when compared to the original study [36, 39]. CMt infection led to anaemia in the prednisolone-treated cats. The cat that received the highest dose of prednisolone (Cat Z) showed the shortest incubation period, the highest blood load, and the lowest PCV value among the three amplificatory cats. An inverse association between the CMt load and the PCV value was found, as has been previously reported for two cats experimentally infected with CMt [36].

Due to the unavailability of culture assays, it could not be determined in vitro whether the CMt DNA in the saliva of infected cats represented viable organisms. According to our findings, however, CMt-positive saliva did not pose a major risk of infection to naïve cats. Nonetheless, one cat (Cat 2) seroconverted following the oral inoculation of CMt-positive saliva. Thus, in this cat, it appeared that at least CMt antigens must have been transmitted via saliva. Any resulting CMt replication must have been below the detection limit of the PCR assay or occurred in tissues other than the peripheral blood. It is unlikely that the CMt load in the saliva samples was too low to result in a positive PCR result given that the same copy number administered in blood was sufficient to infect cats following subcutaneous inoculation, even without pre-existing prednisolone treatment.

CMt-positive blood was found to be infectious when administered intravenously [36], intraperitoneally, or subcutaneously (present study). In contrast, the oral inoculation of CMt-positive blood did not result in infection in the present study. In a very early study, the inoculation of 5 mL of blood from haemoplasma-infected cats (determined by microscopy; haemoplasma species not molecularly determined) resulted in infection in the recipient cats [4]. In our opinion, however, it seems highly unlikely that a cat would be exposed to such a large volume of infectious blood under natural conditions. Therefore, in the present study, we did not exceed 500 μL of infectious blood collected at the peak bacteraemia and aimed at simulating a more natural process.

As a model for aggressive interactions, a subcutaneous inoculation was selected. All cats inoculated with as little as two drops (10 μL) of CMt-positive blood became infected. Small volumes of blood from a CMt-positive cat could be transmitted to a recipient cat via a bite. Moreover, this volume corresponds to the volume that has been reported to be consumed by *C. felis* per day during haematophagous activity [3]. Experimental studies of flea transmission have not yet been conclusive [42, 43], and the role of *C. felis* in the transmission of haemoplasmas remains unclear.

The differences observed between the amplificatory and recipient cats over the course of CMt infection (minimum PCV, peak CMt load, incubation period) may be attributable not only to the attempted immunosuppression but also to the different infection route, infection dose, and age of the cats. It cannot be determined whether each of these factors by itself had a significant influence on the infection, but, overall, the shorter the incubation period and the higher the CMt load, the lower the PCV value.

Eight of nine cats that became PCR-positive after subcutaneous CMt exposure showed a similar infection course: they became PCR-positive within 2 to 5 weeks and seroconverted within 5 weeks p.i. and within 2 weeks following a positive blood-PCR result. A somewhat different course of infection was observed

in two cats, and, remarkably, these were siblings and the only two cats in the present study with blood type B. One cat, Cat 2, was already seropositive at the time of subcutaneous exposure because it had seroconverted after oronasal exposure to CMt-positive saliva (see above). Cat 2 only became PCR-positive later in the course of infection, following subcutaneous exposure (day 45 p.i.), and developed a lower peak CMt blood load (1.4×10^3 copies/mL) compared to the other eight cats (median peak load: 5.7×10^4 copies/mL). The second blood type B cat, Cat 7, remained blood PCR-negative, even after repeated CMt exposures. This cat subsequently seroconverted after the second CMt inoculation but was never blood PCR-positive. While the oronasal exposure to CMt antigens and the resulting immune response in Cat 2 might have led to partial protection and a reduced CMt load, the antibiotic treatment of Cat 7 three weeks before the first CMt blood inoculation may have influenced the course of infection. Alternatively, the blood type of the two cats, both blood type B, or other genetic factors may have played a role in their susceptibility to CMt. However, due to the small number of subjects in the present study, this cannot be determined. This course of CMt infection described herein, i.e., seroconversion in the absence of CMt in the peripheral blood, as detected by real-time PCR, has not been described previously.

The microscopy of blood smears to diagnose CMt infection is not recommended, which is consistent with earlier findings [32, 37]. We assume that the inability to detect CMt via this method may be due to the low CMt blood load [37], which was found to be significantly lower than the Mhf blood load [37]. Even the peak CMt blood load (10^6 copies/mL) corresponded to only one CMt copy per 10^3 to 10^4 erythrocytes, a number that is undetectable by routine light microscopy.

The serological assay described in this study, based on an HspA1 fragment of Mhf detected via western blot, demonstrated seroconversion in all 13 cats exposed to CMt. Using this assay, two seropositive but PCR-negative animals were identified. Among the cats administered antibiotics, the intensity of the western blot band decreased and/or vanished over time, while the ten untreated cats remained seropositive. The antibiotic treatment may have cleared CMt from the blood and tissues, whereas a sequestered CMt infection may have led to the continuous low-level stimulation of the humoural immune response in untreated cats. The serological assay described in this study may be an excellent tool to further investigate the pathogenesis of haemoplasma infections.

In conclusion, our results indicate that social contact, such as the sharing of food dishes or grooming, with CMt-positive cats is unlikely to pose a risk of infection to naïve cats. However, our hypothesis was partially confirmed as the direct transmission of CMt via aggressive interaction (e.g. cat bite) may occur if the recipient cat is exposed to a small volume of infectious blood.

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REFERENCES

[1] Alleman A.R., Pate M.G., Harvey J.W., Gaskin J.M., Barbet A.F., Western immunoblot analysis of the antigens of *Haemobartonella felis* with sera from experimentally infected cats, J. Clin. Microbiol. (1999) 37:1474–1479.

[2] Dean R.S., Helps C.R., Gruffydd Jones T.J., Tasker S., Use of real-time PCR to detect *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' in the saliva and salivary glands of haemoplasma-infected cats, J. Feline Med. Surg. (2008) 10:413–417.

[3] Dryden M.W., Gaafar S.M., Blood consumption by the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae), J. Med. Entomol. (1991) 28:394–400.

[4] Flint J.C., Roepke M.H., Jensen R., Feline infections anemia. II. Experimental cases, Am. J. Vet. Res. (1959) 20:33–40.

[5] Foley J.E., Harrus S., Poland A., Chomel B., Pedersen N.C., Molecular, clinical, and pathologic

comparison of two distinct strains of *Haemobartonella felis* in domestic cats, Am. J. Vet. Res. (1998) 59:1581–1588.

[6] Foley J.E., Pedersen N.C., '*Candidatus* Mycoplasma haemominutum', a low-virulence epierythrocytic parasite of cats, Int. J. Syst. Evol. Microbiol. (2001) 51:815–817.

[7] Fujihara M., Watanabe M., Yamada T., Harasawa R., Occurrence of '*Candidatus* Mycoplasma turicensis' infection in domestic cats in Japan, J. Vet. Med. Sci. (2007) 69:1061–1063.

[8] Gary A.T., Richmond H.L., Tasker S., Hackett T.B., Lappin M.R., Survival of *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' in blood of cats used for transfusions, J. Feline Med. Surg. (2006) 8:321–326.

[9] George J.W., Rideout B.A., Griffey S.M., Pedersen N.C., 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. (2002) 63:1172–1178.

[10] Gomes-Keller M.A., Gonczi E., Tandon R., Riondato F., Hofmann-Lehmann R., Meli M.L., Lutz H., Detection of feline leukemia virus RNA in saliva from naturally infected cats and correlation of PCR results with those of current diagnostic methods, J. Clin. Microbiol. (2006) 44:916–922.

[11] Grindem C.B., Corbett W.T., Tomkins M.T., Risk factors for *Haemobartonella felis* infection in cats, J. Am. Vet. Med. Assoc. (1990) 196:96–99.

[12] Gut M., Leutenegger C.M., Huder J.B., Pedersen N.C., Lutz H., One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses, J. Virol. (1999) 77:37–46.

[13] Helps C., Lait P., Tasker S., Harbour D., Melting curve analysis of feline calicivirus isolates detected by real-time reverse transcription PCR, J. Virol. Methods (2002) 106:241–244.

[14] Helps C., Reeves N., Egan K., Howard P., Harbour D., Detection of *Chlamydophila felis* and feline herpesvirus by multiplex real-time PCR analysis, J. Clin. Microbiol. (2003) 41:2734–2736.

[15] Hoelzle K., Grimm J., Ritzmann M., Heinritzi K., Torgerson P., Hamburger A., et al., Use of recombinant antigens to detect antibodies against *Mycoplasma suis*, with correlation of serological results to hematological findings, Clin. Vaccine Immunol. (2007) 14:1616–1622.

[16] Hoelzle L.E., Hoelzle K., Ritzmann M., Heinritzi K., Wittenbrink M.M., *Mycoplasma suis* antigens recognized during humoral immune response in experimentally infected pigs, Clin. Vaccine Immunol. (2006) 13:116–122.

[17] Hoelzle L.E., Hoelzle K., Harder A., Ritzmann M., Aupperle H., Schoon H.A., et al., First identification and functional characterization of an immunogenic protein in unculturable haemotrophic Mycoplasmas (*Mycoplasma suis* HspA1), FEMS Immunol. Med. Microbiol. (2007) 49:215–223.

[18] Lappin M.R., Griffin B., Brunt J., Riley A., Burney D., Hawley J., et al., Prevalence of Bartonella species, haemoplasma species, Ehrlichia species, *Anaplasma phagocytophilum*, and *Neorickettsia risticii* DNA in the blood of cats and their fleas in the United States, J. Feline Med. Surg. (2006) 8:85–90.

[19] Leutenegger C.M., Klein D., Hofmann-Lehmann R., Mislin C., Hummel U., Boni J., et al., Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system, J. Virol. (1999) 78:105–116.

[20] Lobetti R.G., Tasker S., Diagnosis of feline haemoplasma infection using a real-time PCR assay, J. S. Afr. Vet. Assoc. (2004) 75:94–99.

[21] Luria B.J., Levy J.K., Lappin M.R., Breitschwerdt E.B., Legendre A.M., Hernandez J.A., et al., Prevalence of infectious diseases in feral cats in Northern Florida, J. Feline Med. Surg. (2004) 6:287–296.

[22] Meli M., Kipar A., Muller C., Jenal K., Gonczi E., Borel N., et al., High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats, J. Feline Med. Surg. (2004) 6:69–81.

[23] Messick J.B., Berent L.M., Cooper S.K., Development and evaluation of a PCR-based assay for detection of *Haemobartonella felis* in cats and differentiation of *H. felis* from related bacteria by restriction fragment length polymorphism analysis, J. Clin. Microbiol. (1998) 36:462–466.

[24] Molia S., Chomel B.B., Kasten R.W., Leutenegger C.M., Steele B.R., Marker L., et al., Prevalence of Bartonella infection in wild African lions (*Panthera leo*) and cheetahs (*Acinonyx jubatus*), Vet. Microbiol. (2004) 100:31–41.

[25] Neimark H., Johansson K.E., Rikihisa Y., Tully J.G., Proposal to transfer some members of the genera Haemobartonella and Eperythrozoon to the genus Mycoplasma with descriptions of '*Candidatus* Mycoplasma haemofelis', '*Candidatus* Mycoplasma haemomuris', '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii', Int. J. Syst. Evol. Microbiol. (2001) 51:891–899.

[26] Osterhaus A.D., Horzinek M.C., Reynolds D.J., Seroepidemiology of feline infectious peritonitis virus infections using transmissible gastroenteritis virus as antigen, Zentralbl. Veterinarmed. B (1977) 24:835–841.

[27] Rikihisa Y., Kawahara M., Wen B., Kociba G., Fuerst P., Kawamori F., et al., Western immunoblot analysis of *Haemobartonella muris* and comparison of 16S rRNA gene sequences of *H. muris*, *H. felis*, and *Eperythrozoon suis,* J. Clin. Microbiol. (1997) 35: 823–829.

[28] Shaw S.E., Kenny M.J., Tasker S., Birtles R.J., Pathogen carriage by the cat flea *Ctenocephalides felis* (Bouche) in the United Kingdom, Vet. Microbiol. (2004) 102:183–188.

[29] Sykes J.E., Drazenovich N.L., Ball L.M., Leutenegger C.M., Use of conventional and real-time polymerase chain reaction to determine the epidemiology of hemoplasma infections in anemic and nonanemic cats, J. Vet. Intern. Med. (2007) 21:685–693.

[30] Tandon R., Cattori V., Gomes-Keller M.A., Meli M.L., Golder M.C., Lutz H., Hofmann-Lehmann R., Quantitation of feline leukaemia virus viral and proviral loads by TaqMan real-time polymerase chain reaction, J. Virol. Methods (2005) 130:124–132.

[31] Taroura S., Shimada Y., Sakata Y., Miyama T., Hiraoka H., Watanabe M., et al., Detection of DNA of 'Candidatus Mycoplasma haemominutum' and Spiroplasma sp. in unfed ticks collected from vegetation in Japan, J. Vet. Med. Sci. (2005) 67:1277–1279.

[32] Tasker S., Binns S.H., Day M.J., Gruffydd-Jones T.J., Harbour D.A., Helps C.R., et al., Use of a PCR assay to assess the prevalence and risk factors for M*ycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' in cats in the United Kingdom, Vet. Rec. (2003) 152:193–198.

[33] Tasker S., Helps C.R., Day M.J., Gruffydd-Jones T.J., Harbour D.A., Use of real-time PCR to detect and quantify *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' DNA, J. Clin. Microbiol. (2003) 41:439–441.

[34] Tasker S., Braddock J.A., Baral R., Helps C.R., Day M.J., Gruffydd-Jones T.J., Malik R., Diagnosis of feline haemoplasma infection in Australian cats using a real-time PCR assay, J. Feline Med. Surg. (2004) 6:345–354.

[35] Vogtlin A., Fraefel C., Albini S., Leutenegger C.M., Schraner E., Spiess B., et al., Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR, J. Clin. Microbiol. (2002) 40:519–523.

[36] Willi B., Boretti F.S., Cattori V., Tasker S., Meli M.L., Reusch C., et al., Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland, J. Clin. Microbiol. (2005) 43:2581–2585.

[37] Willi B., Boretti F.S., Baumgartner C., Tasker S., Wenger B., Cattori V., et al., Prevalence, risk factor analysis, and follow-up of infections caused by three feline hemoplasma species in cats in Switzerland, J. Clin. Microbiol. (2006) 44:961–969.

[38] Willi B., Tasker S., Boretti F.S., Doherr M.G., Cattori V., Meli M.L., et al., Phylogenetic analysis of '*Candidatus* Mycoplasma turicensis' isolates from pet cats in the United Kingdom, Australia, and South Africa, with analysis of risk factors for infection, J. Clin. Microbiol. (2006) 44:4430–4435.

[39] Willi B., Boretti F.S., Meli M.L., Bernasconi M.V., Casati S., Hegglin D., et al., Real-time PCR investigation of potential vectors, reservoirs and shedding patterns of feline hemotropic mycoplasmas, Appl. Environ. Microbiol. (2007) 73:3798–3802.

[40] Willi B., Boretti F.S., Tasker S., Meli M.L., Wengi N., Reusch C.E., et al., From Haemobartonella to hemoplasma: molecular methods provide new insights, Vet. Microbiol. (2007) 125:197–209.

[41] Willi B., Filoni C., Catao-Dias J.L., Cattori V., Meli M.L., Vargas A., et al., Worldwide occurrence of feline hemoplasma infections in wild felid species, J. Clin. Microbiol. (2007) 45:1159–1166.

[42] Woods J.E., Brewer M.M., Hawley J.R., Wisnewski N., Lappin M.R., Evaluation of experimental transmission of '*Candidatus* Mycoplasma haemominutum' and *Mycoplasma haemofelis* by *Ctenocephalides felis* to cats, Am. J. Vet. Res. (2005) 66:1008–1012.

[43] Woods J.E., Wisnewski N., Lappin M.R., Attempted transmission of '*Candidatus* Mycoplasma haemominutum' and *Mycoplasma haemofelis* by feeding cats infected *Ctenocephalides felis,* Am. J. Vet. Res. (2006) 67:494–497.