Evaluation of experimental transmission of *Candidatus* Mycoplasma haemominutum and *Mycoplasma haemofelis* by *Ctenocephalides felis* to cats

James E. Woods, DVM; Melissa M. Brewer; Jennifer R. Hawley; Nancy Wisnewski, PhD; Michael R. Lappin, DVM, PhD

Objective—To determine whether *Ctenocephalides felis* can transmit *Mycoplasma haemofelis* (Mhf) and *Candidatus* Mycoplasma haemominutum (Mhm) through hematophagous activity between cats.

Animals—11 cats.

Procedure—2 cats were carriers of either Mhf or Mhm. Nine cats had negative results via polymerase chain reaction (PCR) assay for Mhf and Mhm DNA; 3 of those cats were infected from the chronic carriers via IV inoculation of blood. At the time of maximum organism count for each of the *Mycoplasma* spp, 1 chamber containing 100 *C felis* was bandaged to the amplifier cats. Five days later, fleas, feces, larvae, or eggs from each chamber were analyzed for *Mycoplasma* spp DNA. Viable fleas from the chambers were allocated into new chambers (3 Mhm and 6 Mhf) and attached to naïve cats for 5 days. Cats were monitored daily for clinical signs and weekly via CBC and PCR assay for infection with Mhf or Mhm for a minimum of 8 weeks.

Results—Uptake of Mhf and Mhm DNA into fleas, feces, and, potentially, eggs and larvae was detected. Of the naïve cats fed on by Mhf-infected fleas, 1 cat transiently yielded positive PCR assay results for Mhf on 1 sampling date without clinical or hematologic changes consistent with Mhf infection.

Conclusions and Clinical Relevance—Results suggest that hematophagous transfer of Mhm and Mhf into fleas occurred and that *C felis* is a possible vector for Mhf via hematophagous activity. (*Am J Vet Res* 2005;66:1008–1012)

Feline hemoplasmosis is caused by 2 distinct *Mycoplasma* spp: *Candidatus* Mycoplasma **haemominutum** (**Mhm**; previously referred to as the small form or California variant of *Haemobartonella felis*) and **Mycoplasma haemofelis** (**Mhf**; previously referred to as the large form or Ohio variant of *H felis*).[1,2]

Clinical signs associated with these infections can range from a fulminate, potentially fatal, hemolytic crisis to nonspecific waxing and waning signs including lethargy, anorexia, and fever. In experimental infection studies,[3] Mhf usually induces anemia and associated clinical signs, whereas Mhm usually induces subtle, nonspecific signs of infection. In 1 experimental study,[4] dual infections with both organisms resulted in more severe illness than infection with either organism alone. More substantial abnormalities are associated with Mhm when FeLV coinfection occurs.[5]

The inability to culture the organism in vitro has limited the number of effective diagnostic strategies. Traditionally, diagnosis has relied upon appropriate clinical signs combined with cytologic identification of the organism on a blood smear by use of light microscopy. Recently, **polymerase chain reaction (PCR)** assays have become commercially available that are more sensitive and specific than cytologic examination and have been used to reveal the prevalence of infection to be more common than previously estimated. In 1 study[6] of 220 cats with or without clinical signs of hemoplasmosis, 10 (4.5%) cats were infected with Mhm, 28 (12.7%) were infected with Mhf, and 5 (2.3%) were infected with both organisms with an overall prevalence of 19.5%. In a study[7] in the United Kingdom, the overall prevalence rate in 426 cats infected with either or both *Mycoplasma* spp was 18.5%.

Experimental transmission with infected blood has been attained via the IV, IP, and PO routes, but most studies were performed before the 2 species were recognized.[8] It has been hypothesized that blood-sucking arthropods may be involved in the natural transmission of the organisms because hemoplasmosis is common in geographic areas with large flea burdens. However, other recent work[6] has revealed high prevalence rates of hemoplasmosis even in areas with low flea burdens.

In support of the possible role of fleas in the transmission of these organisms, DNA of both Mhf and Mhm was recently amplified from fleas collected from client-owned animals by use of a PCR assay.[a] In another study[b] of cats used as blood donors, *Mycoplasma* spp were detected in 9.8% of the cats tested; the prevalence rate was 20.1% in cats with a known history of flea exposure. These findings suggest that fleas ingest both *Mycoplasma* spp while feeding; further data are needed to evaluate whether *Ctenocephalides felis* is a vector for the organisms. The objective of the study reported here

was to assess the ability of *C felis* to transmit Mhf and Mhm from infected cats to naïve cats solely through hematophagous activity.

Materials and Methods

The protocol used in this study was reviewed and approved by the Colorado State University Animal Care and Use Committee in accordance with federal regulations. Upon completion of the study, all cats were adopted to private owners.

Mycoplasma spp PCR assay—All cat and flea samples assessed in this study were assayed by use of the same PCR assay that amplifies both Mhf and Mhm.[6] Blood in EDTA was digested for DNA extraction and assayed, as described, after a maximum of 4 days' storage at 4°C. Fleas were sorted into 4 categories as follows: viable males, nonviable males, viable females, and nonviable females. From each category, fleas in groups of 5 were assayed together. In instances in which there were < 5 fleas available, these were assayed in groups of < 5 or in a group of 6 when 1 flea remained. Random, and not quantified, amounts of flea feces, flea feces and eggs, or flea feces and larvae were assayed together. Removal of residue of feces from fleas, eggs, or larvae was not attempted. These samples were individually placed in a 1.7-mL microcentrifuge tube and frozen at –70°C for 5 minutes. Upon removal from –70°C storage, the tube was placed on dry ice and the contents were thoroughly pulverized with a pestle, to which a genomic DNA isolation reagent[c] (30 µL/flea) was added. The resultant mixture was heated for 3 minutes at 95°C and centrifuged at 16,000 $\times$ g for 3 minutes to create a pellet of the flea components. The supernatant was placed in a separate 1.7-mL microcentrifuge tube to which 15 µL of 100% ethanol/flea was added, and the combination vortexed briefly. The mixture was then centrifuged at 16,000 $\times$ g for 5 minutes at 20°C. The supernatant was discarded, and 150 µL of cold 70% ethanol was added to the DNA pellet and centrifuged for 5 minutes at 16,000 $\times$ g. The supernatant was removed and discarded, and the resultant pellet was air-dried for 30 minutes. The DNA was suspended in 30 to 100 µL of water. Five microliters of the resultant mixture was then assayed by use of the PCR assay.[6] A negative control consisting of water and a positive control consisting of blood from known laboratory-maintained carriers of both *Mycoplasma* spp being studied were assayed with all experimental samples.

Cats—Eleven domestic shorthair cats (5 male and 6 female) from the ages of 1 to 3 years were used in this study. The cats were obtained from a commercial vendor, lacked physical examination abnormalities as determined by a licensed veterinarian, and were free of ticks and fleas. All cats had negative results of tests for FeLV antigen,[d] FIV antibodies,[d] and *Bartonella* spp DNA by use of PCR assay.[9] All cats to be experimentally infected were revealed by use of PCR assay to have negative results for DNA of both *Mycoplasma* spp[6] from 1 to 3 times at weekly intervals before the start of the study. Multiple *Mycoplasma* DNA PCR assays were performed at weekly intervals on selected cats to eliminate false negatives attributable to peracute *Mycoplasma* infection or cyclical low organism counts, although both of these scenarios seemed unlikely because none of the cats had opportunity for exposure to either *Mycoplasma* sp. Concurrent with these PCR assays, for all cats, CBCs were also performed from 1 to 3 times at weekly intervals. All cats were fed a dry canned commercial feline diet and fresh water ad libitum.

Cats used in this study were referred to by group numbers as follows: group 1 included cats (n = 2) that were chronic carriers of either Mhm or Mhf, group 2 included cats (3) used to amplify the *Mycoplasma* infection from group 1 cats (1 cat used to amplify Mhm and 2 cats used to amplify Mhf), group 3 included *Mycoplasma*-naïve cats (3) that were

fed on by Mhm-infected fleas, and group 4 included *Mycoplasma*-naïve cats (6) fed on by Mhf-infected fleas.

Primary infection—The strains of Mhf and Mhm used in this study were initially isolated from naturally infected cats[4] and were maintained by group 1 cats inoculated approximately 20 months previously. The group 1 cats were otherwise healthy, nonanemic, nonfebrile, and receiving no medications. To amplify the infections, 2 mL of heparinized blood from the Mhm-carrier group 1 cat was inoculated IV into a *Mycoplasma* spp–negative study cat in group 2, and heparinized blood from the Mhf-carrier group 1 cat was inoculated IV (2 mL/cat) into 2 of the *Mycoplasma* spp–negative study cats in group 2. Thus, group 2 contained 1 cat inoculated IV with Mhm and 2 cats inoculated IV with Mhf. Group 2 cats were observed for clinical signs consistent with transfusion reaction, including tachypnea and lethargy, after the inoculation. The cats in groups 1 and 2 were group-housed separately from the remainder of the cats used in the study. Group 2 cats were monitored daily for clinical signs consistent with infection, including rectal temperature, attitude, and mucous membrane coloration, in addition to being monitored weekly for infection by use of CBC and PCR assay for *Mycoplasma* spp DNA for the duration of the study.

Fleas and flea chambers—The fleas[e] used in these experiments were laboratory-reared, unfed adult fleas that were captured just after emergence from their pupae and raised in an indoor environment without exposure to animals. The adult fleas used to produce the fleas used in the study had only been fed bovine blood, a species that neither *Mycoplasma* spp is known to infect. Fleas were allowed to feed on subject cats with the use of flea chambers and chamber attachment techniques described in the literature.[10] Chambers were loaded with fleas, and the postfeeding chambers opened only at the Heska facility and not within the confines of the study facility. One loaded flea chamber per cat was bandaged to the shaved lateral portion of the thorax of the study cats. Chamber position and bandage tightness were monitored daily. All fleas were killed by hypothermia by placing them in –70°C until assayed. Prior to the beginning of the study, 7 fleas from the group to be used in this study yielded negative results for *Mycoplasma* spp DNA via PCR assay.[6]

Mhm—The Mhm-inoculated cat in group 2 had positive results of PCR assay on day 24 **postinoculation (PI)** and had consistent positive PCR assay results for the duration of the study. On day 28 PI, which was approximately the time for maximum organism count for Mhm as detected by real-time PCR assay,[f] a flea chamber containing approximately 100 fleas of approximately equal male-to-female ratio was bandaged to this cat and left in place until day 33 PI. The chamber contents were sorted and analyzed as described, and a random sample of viable fleas was retained for PCR assay analysis. The remaining viable fleas were equally divided and placed in 3 new chambers of approximately 25 fleas/chamber, which were immediately bandaged to the group 3 cats for a 5-day feeding period. After this time, the chambers were removed and the contents analyzed by PCR assay, as described. Blood (approx 1.5 mL) for CBC and PCR assay was collected from each cat in group 3 and placed in tubes that contained EDTA on days 0, 7, 14, 21, 28, 35, 42, 49, and 56 after exposure to fleas. All group 3 cats were monitored daily for rectal temperature, attitude, and mucous membrane coloration for the duration of the study.

Mhf—The Mhm experiment was completed prior to performing the Mhf experiment. None of the cats in group 3 had positive results of Mhm PCR assay by day 56 after exposure to fleas. Thus, the 3 cats in group 3 as well as 3 additional cats naïve to *Mycoplasma* spp were used in this experiment.

These 6 Mycoplasma-naïve cats made up group 4. The 2 Mhf-inoculated group 2 cats (C044 and G099) yielded positive results of PCR assay by day 8 PI and for the duration of the study. A flea chamber containing approximately 100 fleas of approximately equal male-to-female ratio was attached to both of these cats on day 11 PI and remained in place until day 16 PI because maximum organism count with Mhf usually occurs on approximately day 14, as determined by use of real-time PCR assay.[11] Upon removal of the chambers, it was discovered that only 15 fleas remained viable in one of the chambers. The chamber had slipped on the cat (G099), resulting in starvation of most of the fleas. It was decided that 15 fleas would not be an adequate number to divide between the 3 new chambers, so these 15 fleas were analyzed by use of PCR assay. A new chamber loaded with approximately 100 fleas of approximately equal male-to-female ratio was placed on the cat (G099) from day 16 through day 21 PI. Upon removal of the chambers from the Mhf-inoculated group 2 cats, the contents were sorted and analyzed as described, and a random sample of viable fleas was retained for PCR assay analysis. The remaining viable fleas from each chamber were equally divided into 6 new chambers of approximately 25 fleas/chamber and attached to the 6 cats in group 4 for a 5-day feeding period. At the time of chamber removal from the group 4 cats, the contents were sorted and analyzed by use of PCR assay, as described. Blood (approx 1.5 mL) for CBC and PCR assay was collected and placed in EDTA from the 3 cats in group 4 that received the flea chamber on day 16 PI on days 0, 10, 17, 24, 34, 39, 45, 52, 59, and 66 after exposure to infected fleas and from the 3 cats in group 4 that received the flea chamber on day 21 PI on days 0, 5, 12, 19, 29, 34, 40, 47, 54, and 64 after exposure to infected fleas. All cats in group 4 were monitored daily for rectal temperature, attitude, and mucous membrane coloration for the duration of the study.

Results

All cats in group 2 had positive PCR assays with varying PCVs (**Figure 1**). The largest decreases in PCV were seen in the Mhf-inoculated cats, with both cats becoming anemic as defined by the Colorado State University PCV reference range of 30% to 46%; the Mhf-infected cats had PCVs at 1 sampling point of 21% and 25%, respectively. In contrast, the Mhm-inoculated cat's PCV never decreased to < 35%. Additionally, both of the Mhf-inoculated cats had clinical signs of illness including lethargy, and 1 of these cats became febrile with a rectal temperature of 39.6°C.

When the flea chamber was removed from the Mhm-inoculated group 2 cat on day 33 PI, all samples of fleas (viable or nonviable), larvae, feces, or eggs yielded positive PCR results for Mhm DNA. After the remainder of the viable fleas was divided and allowed to feed on the group 3 cats, some of the flea chamber
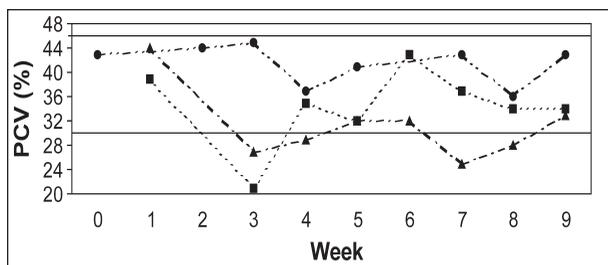

Figure 1—Values for PCV in 3 cats at various weeks after IV inoculation (1 cat each; time point 0) with *Candidatus* Mycoplasma haemominutum (circles) or *Mycoplasma haemofelis* (squares, triangles). Solid lines indicate reference range.

contents from each cat yielded positive results for PCR assay for Mhm DNA. However, during the 56-day monitoring period, none of the group 3 cats yielded positive results for Mhm DNA in blood and none of those cats became anemic.

When the chambers were removed from the Mhf-inoculated group 2 cats on day 16 PI, all 15 viable fleas from the slipped chamber yielded positive results of PCR assay for Mhf DNA. The new chamber that was attached to this cat (G099) was then removed on day 21 PI, and most of the samples of fleas (viable or non-viable), larvae, feces, or eggs from this chamber yielded positive results of PCR assay for Mhf DNA. However, a higher percentage of dead fleas was in this chamber, compared with fleas that fed on the Mhm-inoculated group 2 cat. Although all of the larvae, eggs, and fecal samples tested from the chamber removed on day 16 PI from the other cat yielded positive results of PCR assay for Mhf DNA, only 1 of the 3 adult flea samples tested yielded positive results. After the remainder of the fleas was divided and allowed to feed on the 6 group 4 cats, selected samples from chambers on 4 of the 6 cats yielded positive results of PCR assay for Mhf.

During the 66-day monitoring period, 1 cat transiently yielded positive results of PCR assay for Mhf in its blood 12 days after flea chamber attachment. A second PCR assay was performed on this same aliquot of blood, which also yielded positive results for Mhf DNA. Negative control samples for both of these assays yielded negative results, suggesting that cross-contamination of samples during the assays did not occur. Blood from this cat yielded negative results of PCR assay on all subsequent sample dates, and anemia never developed. In addition, clinical signs of infection, including pale mucous membranes, fever, lethargy, and decreased appetite, did not develop in that cat. The remaining group 4 cats yielded negative results of PCR assay for the entire study and never became anemic.

Discussion

Intravenous inoculation of blood from known carriers of Mhf and Mhm (group 1) into the naïve cats (group 2) described here resulted in infection.[4,12] The Mhf-inoculated group 2 cats became anemic, whereas the Mhm-inoculated group 2 cat did not. This finding was similar to that of other studies[4,12] that used those strains of the *Mycoplasma* spp and the IV inoculation protocol and revealed Mhf to be more pathologic than Mhm.

The fleas used in these experiments were laboratory bred and raised with no opportunity for exposure to either *Mycoplasma* sp. Also, a sample of the fleas yielded negative results for DNA of both *Mycoplasma* spp prior to the start of the experiments. Therefore, the detection of either Mhf or Mhm DNA in the fleas allowed to feed on infected cats revealed that DNA of both organisms entered *C felis* during the ingestion of a blood meal. Fleas that feed on cats naturally exposed to Mhf and Mhm also yield DNA of both organisms.[a] In our study, DNA of both *Mycoplasma* spp was detected in flea feces, but further experiments will be required to determine whether the organisms are passed in feces alive, as occurs with *Bartonella henselae*.[13,14] Some flea

eggs and larvae also yielded positive results of PCR assay for DNA of both *Mycoplasma* spp. However, because we did not attempt to remove all residues of flea feces from the flea eggs and larvae, transovarial passage of Mhf or Mhm DNA through *C felis* could not be determined.

One of the group 4 cats that was fed on by Mhf-infected fleas transiently yielded positive results for Mhf DNA on 1 sampling date. The positive result was confirmed by repeating the assay on the same sample of blood. Contamination during the PCR assay was unlikely because the negative controls yielded negative results in the initial and repeated assay. Thus, we believe this indicated that *C felis* can transmit Mhf via hematophagous activity. It was noteworthy that not only was the infection only transiently detected by use of PCR assay but also that clinical or hematologic signs consistent with Mhf infection did not develop in the cat. There are 2 potential explanations for this finding. First, it is possible that Mhf was transmitted to the cat and that an active infection occurred, but the cat was subsequently able to eliminate the infection. This hypothesis could be further explored by detection of serum antibodies against *Mycoplasma* spp by use of an immunofluorescent antibody assay. Second, it is possible that although *C felis* may be a viable mechanical vector for Mhf, it is not the required biological vector that may confer virulence to the organism and aid in establishing a persistent infection.

There are several potential explanations for the observation that *Mycoplasma* spp DNA was not detected in the blood of the other 8 cats exposed to *Mycoplasma*-infected fleas. These include the following: the cats became infected but at a level below the sensitivity limit of the PCR assay, the cats were previously exposed to *Mycoplasma* spp and so were inherently immune, the cats were exposed to both *Mycoplasma* spp by hematophagous activity and successfully infected but eliminated the infections between blood sampling dates, the study design was not optimal for transmission of *Mycoplasma* spp by *C felis*, there was another route of transmission for *Mycoplasma* spp by *C felis*, and inherent characteristics of the *Mycoplasma* spp or flea strain affected transmission.

It is possible that the other cats exposed to *Mycoplasma*-infected fleas became infected but at levels not detectable by our PCR assay. However, we believe this is unlikely because of the sensitivity limit of the assay[6] and the fact that naturally infected cats and cats experimentally inoculated IV yield positive results of this PCR assay for months to years. It is doubtful that the cats used here were previously infected at an undetectable level and were therefore immune because they were laboratory reared without known potential for exposure. These 2 hypotheses could have been explored further by inducing immunosuppression in the cats with glucocorticoids, performing splenectomy, or measuring serum antibodies by use of an immunofluorescent assay before and after exposure to infected fleas.[3]

Several cat and *C felis*-associated variables in the study could have influenced the results. Small numbers of age-matched adult cats were used. Age-acquired resistance to infection is recognized with several infectious feline diseases, including infection with FeLV; it is possible that younger cats are more susceptible to transmission of *Mycoplasma* spp by *C felis*. However, DNA of both Mhf and Mhm has been detected in naturally infected cats of various ages, which makes this hypothesis less likely. In this study, the initial day and duration of attachment of the flea chamber to the cats used to amplify the organisms were chosen to overlap with the occurrence of predicted maximum organism count. It is known that after finding a suitable host, *C felis* feeds on blood, digests it, and excretes feces several times daily. Therefore, flea attachment during maximum organism count should have theoretically provided the optimum time frame for organism uptake in fleas and subsequent transmission to naïve cats. Hence, the flea attachment time and feeding duration used in this study should have maximized the opportunity for transmission from infected to naïve cats. It is possible that *Mycoplasma* spp only survive in fleas for a short period and that successful transmission of these organisms is dependent on an infected flea feeding on a naïve host more rapidly than our study allowed. This could be evaluated by transferring fleas to naïve cats at various intervals after placing them on infected cats. Lastly, fleas are highly dependent on narrow ranges of temperature and relative humidity for survival and maximal reproduction.[15] The longevity of *C felis* increases in environments with a relative humidity approaching 100%. The life cycle of *C felis* is negatively impacted in environments of lower temperatures and lower relative humidity. The mean temperature and relative humidity of the rooms that contained the cats and fleas of this study were approximately 22.2°C and 25%, respectively. These variations from optimal environmental conditions may have influenced efficacy of transmission of *Mycoplasma* spp, particularly because only small numbers of *C felis* were used.

This experiment assessed the ability of fleas to transmit the organism through hematophagous activity only. Because of the chambered flea-feeding technique used,[10] the cats were unable to groom and ingest the fleas or flea by-products as they would have with a natural flea infestation. The natural vector-oral transmission of other parasitic diseases is well established, for example, in the life cycles of *Dipylidium caninum* with fleas and *Hepatozoon americanum* with ticks. Therefore, it is reasonable to hypothesize that maximal transmission of these *Mycoplasma* spp by *C felis* may be from ingestion of infected fleas and flea by-products, rather than by hematophagous activity of infected fleas alone.

Both of these experiments were conducted with laboratory-maintained organisms (fleas and *Mycoplasma* spp). The *Mycoplasma* strains used in these studies were not naturally occurring strains but strains that had been maintained in the laboratory for several years via serial passage through cats, which may have modified the pathogenicity or transmissibility of the *Mycoplasma* strains. To further evaluate this hypothesis, similar experiments would need to be conducted with *Mycoplasma* spp isolated from a naturally

occurring infection. Similarly, the strains of *C felis* used in these experiments were laboratory bred and raised and have been propagated in an artificial environment through hundreds of generations, which may have caused modifications resulting in poor transmission of *Mycoplasma* spp.

A marked difference in flea mortality rate and organism uptake by *C felis* was observed between the Mhf and Mhm experiments. Possible explanations for this include inappropriate chamber attachment or chamber slippage, resulting in inadequate feeding during the Mhf experiment, which was not encountered during the Mhm experiment; different environmental conditions at the times the 2 experiments were performed; *C felis* mortality rate related to exposure to the Mhf organism; and differences in uptake of Mhm and Mhf by *C felis* because of inherent differences in the *Mycoplasma* organisms.

Chamber-related flea deaths during the Mhf experiment seem an unlikely explanation because consistent techniques, materials, and investigators were used to attach the chambers in each of the experiments. The Mhf and Mhm experiments were performed during different seasons in Colorado, where the climate is not optimal for *C felis* survival. The fleas and flea chambers were transported to and from the study facility for counting and sorting, sometimes in unfavorable outdoor environmental conditions, which potentially contributed to the differences in flea mortality rates between the 2 experiments. Perhaps the most intriguing explanation for the differences seen in flea mortality rate could be related to the effect of the *Mycoplasma* organism itself on *C felis*. Possibly, Mhf may be pathogenic to *C felis*, whereas Mhm is not or is less so. Similarly, the apparent differences in Mhf and Mhm organism uptake by *C felis* may be related to inherent organism differences as well.

Results of this study may provide further support to the hypothesis that *C felis* is associated with *Mycoplasma* spp infections of some cats and thereby support the recommendation for strict flea control in cats when possible.

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