Real-time PCR investigation of potential vectors, reservoirs, and shedding patterns of feline hemotropic mycoplasmas

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Abstract

Three hemotropic mycoplasmas have been identified in pet cats: Mycoplasma haemofelis, "Candidatus Mycoplasma haemominutum," and "Candidatus Mycoplasma turicensis." The way in which these agents are transmitted is largely unknown. Thus, this study aimed to investigate fleas, ticks, and rodents as well as saliva and feces from infected cats for the presence of hemotropic mycoplasmas, to gain insight into potential transmission routes for these agents. DNA was extracted from arthropods and from rodent blood or tissue samples from Switzerland and from salivary and fecal swabs from two experimentally infected and six naturally infected cats. All samples were analyzed with real-time PCR, and some positive samples were confirmed by sequencing. Feline hemotropic mycoplasmas were detected in cat fleas and in a few Ixodes sp. and Rhipicephalus sp. ticks collected from animals but not in ticks collected from vegetation or from rodent samples, although the latter were frequently Mycoplasma coccoides PCR positive. When shedding patterns of feline hemotropic mycoplasmas were investigated, "Ca. Mycoplasma turicensis" DNA was detected in saliva and feces at the early but not at the late phase of infection. M. haemofelis and "Ca. Mycoplasma haemominutum" DNA was not amplified from saliva and feces of naturally infected cats, despite high hemotropic mycoplasma blood loads. Our results suggest that besides an ostensibly indirect transmission by fleas, direct transmission through saliva and feces at the early phase of infection could play a role in the epizootiology of feline hemotropic mycoplasmas. Neither the investigated tick nor the rodent population seems to represent a major reservoir for feline hemotropic mycoplasmas in Switzerland.
Real-Time PCR Investigation of Potential Vectors, Reservoirs, and Shedding Patterns of Feline Hemotropic Mycoplasmas

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Three hemotropic mycoplasmas have been identified in pet cats: Mycoplasma haemofelis, “Candidatus Mycoplasma haemominutum,” and “Candidatus Mycoplasma turicensis.” The way in which these agents are transmitted is largely unknown. Thus, this study aimed to investigate fleas, ticks, and rodents as well as saliva and feces from infected cats for the presence of hemotropic mycoplasmas, to gain insight into potential transmission routes for these agents. DNA was extracted from arthropods and from rodent blood or tissue samples from Switzerland and from salivary and fecal swabs from two experimentally infected and six naturally infected cats. All samples were analyzed with real-time PCR, and some positive samples were confirmed by sequencing. Feline hemotropic mycoplasmas were detected in cat fleas and in a few Ixodes sp. and Rhipicephalus sp. ticks collected from animals but not in ticks collected from vegetation or from rodent samples, although the latter were frequently Mycoplasma coccoides PCR positive. When shedding patterns of feline hemotropic mycoplasmas were investigated, “Ca. Mycoplasma turicensis” DNA was detected in saliva and feces at the early but not at the late phase of infection. M. haemofelis and “Ca. Mycoplasma haemominutum” DNA was not amplified from saliva and feces of naturally infected cats, despite high hemotropic mycoplasma blood loads. Our results suggest that besides an ostensibly indirect transmission by fleas, direct transmission through saliva and feces at the early phase of infection could play a role in the epizootiology of feline hemotropic mycoplasmas. Neither the investigated tick nor the rodent population seems to represent a major reservoir for feline hemotropic mycoplasmas in Switzerland.

The agent formerly known as Haemobartonella felis has recently been reclassified as a hemotropic mycoplasma, and three different species have been characterized in cats: Mycoplasma haemofelis, “Candidatus Mycoplasma haemominutum,” and “Candidatus Mycoplasma turicensis” (2, 9, 18, 19, 27, 34). Infections with feline hemotropic mycoplasmas can induce a fulminant, potentially fatal hemolytic crisis, but the pathogenic potential varies greatly among the three different species.

Some years ago, sensitive PCR assays became available for the specific diagnosis of feline hemotropic mycoplasmas (2, 6, 13), and real-time PCR assays have been developed which allow the differentiation and quantification of the three species (28, 33, 34). In applying PCR-based methods, feline hemotropic mycoplasma infections in pet cats have been diagnosed worldwide (6, 13, 16, 25, 26, 30, 33, 36), and a recent study has documented infections in 12 different wild felid species from three different continents (35). Nevertheless, the epizootiology of hemotropic mycoplasmas is still poorly understood, and the transmission routes are largely unknown. Experimental transmission via intravenous, intraperitoneal, and oral routes using infected blood has been successful (8). However, several studies indicate that blood-sucking arthropods could represent the natural means of transmission among cats. In dogs, Mycoplasma haemocanis (formerly Haemobartonella canis), a canine hemotropic mycoplasma that is very closely related to M. haemofelis, can successfully be transmitted among dogs via the dog tick Rhipicephalus sanguineus (21). Furthermore, “Ca. Mycoplasma haemominutum” DNA was recently reported in unfed Ixodes ovatus ticks collected from three different areas in Japan (24). Other PCR-based studies demonstrated “Ca. Mycoplasma haemominutum” and M. haemofelis DNA in cat fleas (Ctenocephalides felis) collected from experimentally or naturally infected cats (15, 22, 37), and DNA of both hemotropic mycoplasmas was detected in cat flea feces (37). However, an attempt to experimentally transmit M. haemofelis and “Ca. Mycoplasma haemominutum” between cats via the hematophagous activity of C. felis was not conclusive: only one out of six cats fed on by M. haemofelis-PCR-positive fleas transiently turned PCR positive, and clinical or hematological signs consistent with feline infectious anemia did not develop in the cat (37). Furthermore, none of the cats fed on by “Ca. Mycoplasma haemominutum”-PCR-positive fleas yielded PCR-positive results in the blood, and the...
attempt to experimentally transmit *M. haemofelis* or “Ca. Mycoplasma haemominutum” by feeding cats with infected *C. felis* was not successful (38).

The discovery that “Ca. Mycoplasma turicensis” is most closely related to rodent hemotropic mycoplasmas, namely, *Mycoplasma coccoides* and *Mycoplasma haemonuris*, brought up the hypothesis of an interspecies transmission of hemotropic mycoplasmas between rodents and cats (34). In addition, there is evidence for a direct transmission of hemotropic mycoplasmas between rodents and cats. In a recent study, “Ca. Mycoplasma haemominutum” but not *M. haemofelis* was detected by PCR in the saliva and salivary glands of cats experimentally infected with the respective hemotropic mycoplasma (7). Furthermore, male cats and cats with outdoor access were more frequently infected with hemotropic mycoplasmas (17, 25, 33, 36), and a history of cat bite abscesses increased the relative risk for infection (11). Hemotropic mycoplasma infections were even reported in areas where flea or tick infestations are uncommon (13).

The aims of the present study were to investigate fleas, ticks, and rodents as well as saliva and feces from infected cats for the presence of hemotropic mycoplasmas to gain insight into potential transmission routes of these agents. (These studies were conducted by B. Willi in partial fulfillment of the requirements for a Ph.D. degree at the Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.)

### MATERIALS AND METHODS

**Arthropods.** A total of 2,198 ticks and 77 fleas were included in the study (Table 1). The 181 ticks from 39 cats and 66 dogs and the 77 fleas from 21 cats were collected by pet owners and veterinarians in northern Switzerland. Because *Rhipicephalus sanguineus* has been reported as a vector of *M. haemocanis* (21), a collection of 67 *Rhipicephalus* sp. ticks was included in the study; these ticks were derived from southern Switzerland because *Rhipicephalus* sp. is not permanently established north of the Alps. Nucleic acid (NA) from 41 of the latter ticks was extracted during a previous study (3). Additionally, NA was available from 1,950 unfed ticks that had been collected from vegetation in the area around Zurich, Switzerland, by the cloth-dragging method (1), during an unrelated study. The arthropods were stored at −20°C in liquid nitrogen or in ethanol at 4°C until transported to the Clinical Laboratory, University of Zurich, Switzerland. Before NA extraction, the ticks and fleas collected from cats and dogs in northern Switzerland were microscopically identified based on their morphology (5, 31).

**Fleas**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of samples</th>
<th>Source(s) or reference</th>
<th>Pooled for extraction</th>
<th>NA extraction method</th>
<th>No. (%) tested PCR positive for the indicated species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. felis</em></td>
<td>73</td>
<td>17 cats</td>
<td>1–5</td>
<td>DNeasy tissue kit</td>
<td>0</td>
</tr>
<tr>
<td><em>C. canis</em></td>
<td>4</td>
<td>4 cats</td>
<td></td>
<td>DNeasy tissue kit</td>
<td>0</td>
</tr>
</tbody>
</table>

**Rodents**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of samples</th>
<th>Source(s) or reference</th>
<th>Pooled for extraction</th>
<th>NA extraction method</th>
<th>No. (%) tested PCR positive for the indicated species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. terrestris</em></td>
<td>186</td>
<td>Free-living rodents</td>
<td>1</td>
<td>MagNa Pure LC DNA isolation kit</td>
<td>0</td>
</tr>
<tr>
<td><em>Apodemus</em> sp.</td>
<td>45</td>
<td>Free-living rodents</td>
<td></td>
<td>DNeasy tissue kit</td>
<td>0</td>
</tr>
<tr>
<td><em>Microtus</em> sp.</td>
<td>7</td>
<td>Free-living rodents</td>
<td></td>
<td>DNeasy tissue kit</td>
<td>0</td>
</tr>
<tr>
<td><em>M. domesticus</em></td>
<td>7</td>
<td>Free-living rodents</td>
<td></td>
<td>DNeasy tissue kit</td>
<td>0</td>
</tr>
</tbody>
</table>

### TABLE 1. Arthropod and rodent species investigated for the presence of hemotropic mycoplasmas

-a The table shows the species, number, source, and extraction methods used for ticks, fleas, and rodents collected from throughout Switzerland and the numbers and percentages that tested PCR positive for *M. haemofelis*, “Ca. Mycoplasma haemominutum,” “Ca. Mycoplasma turicensis,” and *M. coccoides*.  
-b Reference 20 (Schwarzenbach et al., 2004).  
-c Reference 23 (Stieger et al., 2002).  
-d Collected indoors from a dog-keeping household or directly from a dog.  
-e ND, not determined.
RESULTS

Sample characteristics. All ticks collected from pet animals in northern Switzerland were identified as *Ixodes* sp. (Table 1); the 77 fleas collected from cats included 73 *C. felis* and 4 *Ctenocephalides canis*. The species of the 1,950 ticks collected from the vegetation around Zurich had not been microscopically specified. However, based on a previous study (1) and our own experience (32), we assumed that the unspecified ticks consisted mainly of *Ixodes ricinus* ticks, which were the main species captured by the cloth-dragging method from grassland in this geographical region. All arthropod and rodent samples tested PCR positive for 16S rRNA genes. In some NA samples extracted from ticks and dogs, the 18S rRNA gene was amplified unexpectedly high threshold cycle (CT) values (\( \geq 50 \)) and control and sample dilution.

Inhibition was confirmed by testing a 1:10 dilution of the samples; while a CT value of roughly 3.5 higher is expected for an uninhibited PCR (sample dilution of 1:10), the CT values obtained from our samples were 13 to 29 CT lower after dilution. These samples were therefore assayed in the PCRs following by using a 1:10 dilution.

Feline hemotropic mycoplasmas in blood-sucking arthropods. Arthropods were analyzed by real-time PCR for the presence of hemotropic mycoplasma DNA (Table 1). Three and two fleas collected from animals tested positive with real-time PCR for hemotropic mycoplasmas; all positive samples were extracted from individual arthropods. PCR-positive results for "Ca. Mycoplasma haemominutum" were obtained from 2.7% (95% CI, 0 to 6.4%) of the cat fleas and from 2.8% (95% CI, 0 to 6.6%) of the *Ixodes* sp. ticks collected from Swiss pet cats; both PCR-positive *Ixodes* sp. ticks were fully engorged with blood before being subjected to NA extraction. "Ca. Mycoplasma turicensis" was found in 1 (4.3%; 95% CI 0 to 12.6%) *R. sanguineus* tick collected from southern Switzerland. None of the 1,950 ticks collected from vegetation in the region around Zurich tested positive for hemotropic mycoplasma by PCR. Hemotropic mycoplasmas were more frequently detected in *Ixodes* sp. ticks picked from pet animals than in unfed ticks collected directly from vegetation in Switzerland (\( P = 0.0144 \)).

To confirm the two "Ca. Mycoplasma haemominutum" PCR-positive results with *Ixodes* sp. ticks, 171 bp of the 16S rRNA gene was sequenced and aligned with published "Ca. Mycoplasma haemominutum" (GenBank accession no. DQ157149) and "Ca. Mycoplasma haematoparvum" (GenBank accession no. AY532390); sequences from another host were obtained and submitted to GenBank and given the accession numbers EF175168, EF175169, and EF175170.

These samples were therefore assayed in the PCRs following by using a 1:10 dilution.
The frequency of hemotropic mycoplasma PCR-positive cat fleas in the present study (2.7%) is lower than the sample prevalence recently reported for cat fleas collected from cats in the United Kingdom (22) and in the United States (15). In the United Kingdom study, 16 to 37% of the fleas tested real-time PCR positive for “Ca. Mycoplasma haemominutum,” whereas the U.S. study reported 3.3% “Ca. Mycoplasma haemominutum” PCR-positive results. The lower sample prevalence in the present study could be explained by the fact that most fleas were analyzed individually and not in pools, whereas up to 5 or 14 fleas per cat were pooled for extraction in the United Kingdom and the U.S. study, respectively. Furthermore, the cat fleas in the present study were derived from only 17 cats; if the fleas had been pooled per cat before extraction as performed in the studies mentioned, a prevalence of up to 12% (95% CI, 0 to 27.5%) would have resulted. In addition, hemotropic mycoplasma infections are relatively rare in the Swiss pet cat population (33), which would be in agreement with the low number of hemotropic mycoplasma PCR-positive fleas reported in this study.

We only occasionally detected feline hemotropic mycoplasma DNA in ticks from Switzerland, and all of the almost 2,000 unfed ticks collected directly from vegetation tested PCR negative. This suggests that the tick species under investigation play only a marginal role as reservoirs and vectors of feline hemotropic mycoplasmas in Switzerland. A recent study reported the presence of “Ca. Mycoplasma haemominutum” DNA in unfed I. ovatus ticks in Japan (24), suggesting a trans-stadial transmission of hemotropic mycoplasmas in the latter tick species. I. ricinus and Ixodes hexagonus, but not I. ovatus, have been reported in Switzerland; different Ixodes species may vary in their capability to harbor these agents.

The results obtained so far do not support our hypothesis of an interspecies transmission of hemotropic mycoplasmas between rodents and cats. We had assumed an interspecies transmission because of the close phylogenetic relationship of “Ca. Mycoplasma turicensis” to rodent hemotropic mycoplasmas. Because not all rodent species indigenous to Switzerland were included in this study and because the sample size for some species was rather low, the potential role of rodents in the transmission of feline hemotropic mycoplasmas cannot be definitely ruled out. It should be noted that up to 53% of the samples of the investigated free-living rodent species tested real-time PCR positive for M. coccoides. These results provide the first PCR-based evidence that wild rodents are natural hosts for M. coccoides and that infections with the latter agent are common in at least some rodent species in Switzerland. In conclusion, neither the tick nor rodent populations investigated seem to play a major role as reservoirs for feline he-

**DISCUSSION**

This is the first study to report on hemotropic mycoplasma shedding patterns in saliva and feces in infected cats. In addition, it provides a first insight into the occurrence of hemotropic mycoplasmas in arthropods and free-living rodents in Switzerland.

By monitoring two cats experimentally infected with “Ca. Mycoplasma turicensis,” we demonstrated that hemotropic mycoplasma DNA can be detected in saliva and feces up to 9 weeks after infection. Thus, a direct transmission of feline hemotropic mycoplasmas between cats might indeed play a role in the epizootiology of these agents; direct transmission has recently been suggested, based on the common association of hemotropic mycoplasma infection with male gender, outdoor access, and cat bite abscesses (11, 17, 25, 33, 36). “Ca. Mycoplasma turicensis” was not detectable in saliva or feces of experimentally infected cats at later stages of infection. In addition, all feces and saliva samples from privately owned cats tested PCR negative, although some of these cats showed rather high hemotropic mycoplasma blood loads. This finding could indicate that hemotropic mycoplasmas are excreted in the early phase of infection but to a lesser extent by long-term carriers. Since the hemotropic mycoplasma loads in saliva and feces of “Ca. Mycoplasma turicensis”-infected cats were rather low, it may be assumed that oronasal exposure through mutual grooming or sharing of food dishes is hardly sufficient for transmission. Rather, aggressive interactions among cats involving biting might be necessary for a successful direct transmission of hemotropic mycoplasmas. However, experimental transmission studies must be performed to conclusively demonstrate whether direct cat-to-cat transmission plays a role in the epizootiology of feline hemotropic mycoplasmas.

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In conclusion, neither the tick nor rodent populations investigated seem to play a major role as reservoirs for feline he-
motropic mycoplasmas in Switzerland. Remarkably, we detected “Ca. Mycoplasma turicensis” in feaces and saliva of infected cats during the early phase of infection. Thus, besides an ostensibly indirect transmission by fleas, future studies should also address the possibility of a direct transmission of feline hemotropic mycoplasmas, ideally by means of experimental transmission studies.

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