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Originally published at:
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Abstract

Recently, there has been a growing interest in hemotropic mycoplasmal species (also known as the hemoplasmas), the causative agents of infectious anemia in several mammalian species. In felids, two different hemoplasma species have been recognized: Mycoplasma haemofelis (formerly Haemobartonella felis) and "Candidatus Mycoplasma haemominutum." Recently developed molecular methods have allowed sensitive and specific identification and quantification of these agents in feline blood samples. In applying these methods to an epidemiological study surveying the Swiss pet cat population for hemoplasma infection, we discovered a third novel and unique feline hemoplasma isolate in a blood sample collected from a cat that had exhibited clinical signs of severe hemolytic anemia. This agent was readily transmitted via intravenous inoculation to two specific-pathogen-free cats. One of these cats was immunocompromised by the administration of methylprednisolone acetate prior to inoculation, and this cat developed severe anemia. The other immunocompetent cat showed a moderate decrease in packed cell volume. Additionally, an increase in red blood cell osmotic fragility was observed. Sequencing of the entire 16S rRNA gene of the new hemoplasma isolate and phylogenetic analysis showed that the isolate was most closely related to two rodent hemotropic mycoplasmal species, M. coccoides and M. haemomuris. A quantitative real-time PCR assay specific for this newly discovered agent was developed, which will be a prerequisite for the diagnosis of infections with the new hemoplasma isolate.
Identification, Molecular Characterization, and Experimental Transmission of a New Hemoplasma Isolate from a Cat with Hemolytic Anemia in Switzerland

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Received 22 November 2004/Returned for modification 5 December 2004/Accepted 13 February 2005

Recently, there has been a growing interest in hemotropic mycoplasmal species (also known as the hemoplasmas), the causative agents of infectious anemia in several mammalian species. In felids, two different hemoplasma species have been recognized: Mycoplasma haemofelis (formerly Haemobartonella felis) and “Candidatus Mycoplasma haemominutum.” Recently developed molecular methods have allowed sensitive and specific identification and quantification of these agents in feline blood samples. In applying these methods to an epidemiological study surveying the Swiss pet cat population for hemoplasma infection, we discovered a third novel and unique feline hemoplasma isolate in a blood sample collected from a cat that had exhibited clinical signs of severe hemolytic anemia. This agent was readily transmitted via intravenous inoculation to two specific-pathogen-free cats. One of these cats was immunocompromised by the administration of methylprednisolone acetate prior to inoculation, and this cat developed severe anemia. The other immunocompetent cat showed a moderate decrease in packed cell volume. Additionally, an increase in red cell osmotic fragility was observed. Sequencing of the entire 16S rRNA gene of the new hemoplasma isolate and phylogenetic analysis showed that the isolate was most closely related to two rodent hemotropic mycoplasmal species, Mycoplasma coccoides and M. haemomuris. A quantitative real-time PCR assay specific for this newly discovered agent was developed, which will be a prerequisite for the diagnosis of infections with the new hemoplasma isolate.

Haemobartonella felis, the causative agent of feline infectious anemia, was recently reclassified within a newly defined group of hemotropic mycoplasmal species (also known as the hemoplasmas). Sequencing of the 16S rRNA genes of different feline isolates has resulted in the recognition of two different species, Mycoplasma haemofelis and “Candidatus Mycoplasma haemominutum” (1, 10, 11, 20, 24, 26, 28), which parasitize feline red blood cells (RBC). Experimental infection studies have shown that the two species differ in terms of pathogenicity (10, 36): cats experimentally infected with “Candidatus Mycoplasma haemominutum” exhibit minimal clinical signs and anemia is not usually induced, while M. haemofelis infection often results in severe hemolytic anemia. Since M. haemofelis and “Candidatus Mycoplasma haemominutum” cannot be cultured in vitro, diagnosis until recently has relied upon cytological identification on blood smears (4). However, the development of new molecular methods has facilitated the sensitive and specific identification and quantification of these agents (1, 19, 31), and PCR analysis is now the diagnostic method of choice for hemoplasma infections.

There is still little knowledge of the epidemiology of these agents. Both species have been shown to exhibit worldwide geographical distribution (6, 8, 19, 29, 30, 35), and isolates from three different continents have shown near sequence identities (32). We now unexpectedly identified a third hemoplasma agent in a cat with a clinical history of severe hemolytic anemia. The aim of this study was to characterize the clinical and molecular nature of this new isolate.

(These studies were conducted by B. Willi as partial fulfillment of the requirements for a Ph.D. degree at the Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.)

MATERIALS AND METHODS

Animals and experimental design of the transmission experiment. The new isolate was discovered in cat 946, a 13-year-old male castrated cat presented to the Clinic for Small Animal Internal Medicine, University of Zurich, in December 2002. For the transmission experiment, two 10-year-old specific-pathogen-free male castrated cats, cat 1 and cat 2, were used; both were confirmed to be free from feline hemoplasmas (19, 31). The blood types of the three cats were obtained and tested for compatibility using a RapidVet-H feline test (Medical Solution GmbH, Steinhausen, Switzerland). Fresh heparinized blood from cat 946 for inoculation was not available until 10 months after the acute phase of illness, when 4 ml was intravenously inoculated into cat 1. This animal had received methylprednisolone acetate (10 mg/kg of body weight, intramuscularly) two and one weeks before inoculation to increase the probability of transmission. Cat 2 was inoculated intravenously with 4 ml of heparinized blood freshly collected from cat 1 at post-infection (p.i.) day 35. Blood samples from cats 1 and 2 were collected regularly (for time points, see Fig. 1), and the cats were monitored for body temperature, heart rate, respiratory rate, mucous membrane color, attitude, and appetite.

Hematology and biochemistry. Complete hemograms were performed using a Cell-Dyn 3500 instrument (Abbott, Baar, Switzerland). Giemsa-stained blood smears were made from fresh EDTA-anticoagulated blood. Aggregate reticulocytes were counted after methylene blue staining. Serum biochemistry was performed using a Cobas Integra 700 instrument (Roche Diagnostics, Rotkreuz, Switzerland). Reference ranges are stated as 5% and 95% quantiles.

Osmotic fragility and direct Coombs testing. RBC osmotic fragility (OF) was measured by adding 50 µl of freshly collected EDTA-anticoagulated blood to 5 ml of 0.3%-to-0.9% NaCl solutions. After a 1-h incubation at 37°C, samples were
centrifuged (600 g, 10 min), and the hemoglobin content of the supernatant was determined spectrophotometrically at 546 nm. Reference values were determined using nine healthy cats. For direct Coombs testing, erythrocytes from EDTA-anticoagulated blood samples were washed three times with phosphate-buffered saline, and 100 μl of washed RBC were diluted in 4.9 ml 0.9% NaCl solution. A 25-μl aliquot of the RBC suspension was then incubated at 37°C for 1 h with feline antiglobulin reagent (ICN Biomedicals Inc., Eschwege, Germany) in dilutions ranging from 1:2 to 1:5,600 and evaluated for agglutination. Agglutination in dilutions of <1:8 was reported as negative.

DNA extraction and diagnostic PCR assays. DNA was purified from 200 μl EDTA-anticoagulated blood using a MagnaPure LC DNA Isolation Kit I (Roche Diagnostics). PCR assays for the detection of FeLV, feline immunodeficiency virus, and feline parvovirus were performed as previously reported (14, 17, 19, 21, 23, 31).

**FIG. 1. Transmission experiment.** The charts depict the loads of the newly described isolate (log copy number of DNA template per ml of blood, left y axes, black triangles) and the courses of PCV (%)(right y axes, open squares) in cat 1 (A) and cat 2 (B) over 100 days p.i. (x axes). The reference ranges of PCV are shaded grey. The lower detection limits of the real-time PCR assay (100 copies/ml blood) are indicated by dotted lines.

Development of a real-time PCR specific for the new hemoplasma isolate. To detect the new isolate, specific 16S rRNA forward (5'-GAG GCC GAC CAG ACT GGT CTT AAA G-3') and reverse (5'-CTG GCA CAT AGT TWC TGA CTT A-3') primers and a probe (6-carboxyfluorescein-AAA TTT GAT GGT CTT A-3') were used. The 25-μl PCR mixture comprised 1.25 μl of 2× qPCR Mastermix (Eurogentec, Seraing, Belgium), 880 nM of each primer, 200 nM of probe, and 5 μl of template DNA. Assays were performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems). For absolute quantification, plasmids containing the cloned 16S rRNA genes were generated. Purified DNA was digested with NotI and quantified spectrophotometrically. The DNA template was serially diluted tenfold in a solution of 30 μg/ml of salmon sperm DNA (Invitrogen), aliquoted, and stored at −20°C until use.

**RESULTS**

**Identification of a new hemoplasma isolate.** During an epidemiological study to assess the prevalence of hemoplasma infections in Swiss cats, cat 946 yielded discrepant PCR results. DNA extracted from a blood sample from this cat collected in March 2003 tested positive by conventional (19) but negative by real-time PCR specific for *M. haemofelis* and “Candidatus Mycoplasma haemominutum” (31). Four months prior to presentation, cat 946 had exhibited clinical signs consistent with haemobartonellosis, including lethargy, anorexia, pallor, dyspnea, and weight loss. Examination of blood and urine samples collected at that time revealed signs of intravascular hemolysis with a packed cell volume (PCV) of 12% (reference value, 33% to 45%), leukocytosis (25.6 × 10⁹/liter; reference value, 5.0 × 10⁹ to 18.9 × 10⁹/liter), hyperbilirubinemia (34 μmol/liter; reference value, 0 to 15 μmol/liter), and hemoglobinuria. The anemia became highly regenerative 4 days after first presentation (PCV, 17%; aggregated reticulocyte counts of 201,670/μl; regeneration defined by a count of >60,000/μl). A primary immune-mediated hemolytic anemia was suspected, and the cat was treated with corticosteroids. This treatment was associated with only a transient improvement in the cat’s clinical status. After definitive diagnosis of hemoplasma infection and after blood had been collected for the transmission experiment, cat 946 was treated with oral doxycycline (10 mg/kg/day for 14 days), and its clinical condition improved. RBC OF was increased in samples collected between 12 and 21 months after acute illness (50% hemolysis in 0.68% to 0.75% NaCl; reference range, 0.50% to 0.57% NaCl).

**Transmission experiment.** To gain a first insight into the new hemoplasma’s pathogenic potential, a transmission experiment was conducted. The blood samples collected for transmission tested negative for FeLV, feline immunodeficiency virus, feline coronavirus, and feline parvovirus infection. The inoculum received by cat 1 contained a total of 3.8 × 10⁹ copies of the new hemoplasma isolate. Cat 1 became PCR positive 8 days p.i. and remained positive for 88 days (Fig. 1). It developed anemia, with a drop in PCV from 34% to 17% by day...
9 p.i. (Fig. 1). Mild clinical signs of pallor and lethargy were observed, from which the cat recovered without treatment; the PCV remained below the reference range until day 80 p.i. (Fig. 1). The hemoplasma load was inversely correlated with PCV (r = −0.79; P < 0.0001), with a maximal load of 1.9 × 10^7 copies/ml blood on day 18 p.i. (Fig. 1). RBC OF was measured starting at day 36 p.i. (50% hemolysis in 0.57% NaCl); thereafter, it decreased continuously until day 135 p.i. (50% hemolysis in 0.52% NaCl) and stayed at this value until the end of measurement (day 170 p.i.).

The blood sample used to inoculate cat 2 contained a total of 1.6 × 10^4 copies of the new isolate. Cat 2 became PCR positive 11 days p.i. and stayed positive for 80 days (Fig. 1). The RBC OF of cat 2 continuously increased over the first 31 days p.i. (Fig. 2) and returned to normal values 59 days p.i. The RBC OF from days 0 to 31 p.i. is indicated by an arrow. The complete time courses of the OF cannot be given for cats 1 and 946 because this assay had not been established during their early infection periods.

Molecular characterization of the new isolate. Comparison of the 16S rRNA gene sequences obtained from the blood of cats 1, 2, and 946 with those held in GenBank revealed the following similarities (in descending order): M. coccoides (92%), M. haemomuris (90%), M. haemofelis (88%), M. haemocanis (88%), M. haemolama (83%), M. wenyonii (83%), "Candidatus Mycoplasma kahanei" (83%), "Candidatus Mycoplasma haemominutum" (83%), M. ovis (83%), "Candidatus Mycoplasma haemolophivum" (82%), M. suis (82%), M. fastidiosum (82%), and "Candidatus Mycoplasma haemophilus" (81%).

Construction of a phylogenetic tree confirmed the close relationship of the newly described feline hemoplasma isolate with M. coccoides and M. haemomuris, whereas M. haemofelis was clearly less closely related, and "Candidatus Mycoplasma haemominutum" was only distantly related (Fig. 3).

**DISCUSSION**

A new hemoplasma isolate was identified in a naturally infected cat that had exhibited clinical signs of severe intra-vascular hemolytic anemia. The newly discovered agent also induced anemia in two experimentally infected cats, and decreased PCV was associated with high hemoplasma loads. One of the cats had been immunocompromised with methylprednisolone acetate. This corticosteroid alone is not known to cause a decrease in PCV but, in contrast, has been used to treat aplastic and immune-mediated hemolytic anemia due to its ability to increase the half-lives of RBC by decreasing their splenic removal (27).

The two experimentally infected animals showed mild or no clinical signs, whereas the naturally infected animal developed severe illness. Hemoplasma organisms are often opportunistic in their disease potentials, and the presence of parasites or other infectious agents not readily recognized could have worsened the clinical outcome for cat 946. Immunosuppressive...
agents such as FeLV have been shown to influence the clinical courses of hemoplasma infections (5, 12, 15). The three cats of the present study were free of retroviral infections. However, the lack of an adequate immune response due to iatrogenic immunosuppression in cat 1 could explain why this animal developed a more severe outcome of infection, despite being given a lower dose of hemoplasma organisms, than cat 2. An inadequate immune response could also explain the higher hemoplasma loads detected in cat 1 compared to cat 2, which in turn would further underline the importance of the immune response during hemoplasma infections. Additionally, the cat-to-cat passage of the new isolate, although unexpected, could have led to a decreased pathogenic potential of the agent, as to-cat passage of the new isolate, although unexpected, could have led to a decreased pathogenic potential of the agent, as recently reported for “Candidatus Mycoplasma haemominutum” (12). Cat 2 was infected with cat-passaged material which could also have contributed to the lower hemoplasma load and the milder course of infection observed in this cat.

Cat 946 in the present study was a 13-year-old male castrated animal, and cats 1 and 2 in the transmission experiment were also old male castrated animals. Increasing age and male sex have been reported as factors predisposing for feline hemoplasma infections (13, 16, 25, 29). It is possible that the older male status of the cats in the present study influenced the outcome of the infection with the new hemoplasma isolate.

The mechanisms by which feline hemoplasmas, especially M. haemofelis, induce acute hemolysis in infected cats are not well understood. Maede and Hata (22) claimed a central role for the spleen in sequestrating parasitized RBC and removing attached organisms from the RBC surface. They reported an increase in RBC OF following the first appearance of hemoplasma organisms on the RBC surfaces. Increased RBC OF was also observed in the present study for cats 2 and 946, being most severe in the naturally infected cat 946, which developed the most severe clinical signs. Interestingly, the RBC OF was chronically increased in the latter cat, whereas it returned to normal values in cat 2. In the above-mentioned study (22), cats that had exhibited chronic increased RBC OF also tested persistently positive in direct Coombs testing. Different authors have reported that secondary immunologic responses to erythrocytic antigens are associated with hemoplasma infections (7, 22, 37). Direct Coombs testing was not performed in the clinically ill cat. In the two experimentally infected animals, Coombs testing yielded negative results with several blood samples collected throughout the transmission experiment. However, the moderate and transient increase in RBC OF in cat 2 could still be explained by direct RBC damage by the hemoplasma organisms themselves; RBC OF was directly correlated with the hemoplasma load in cat 2, and 1 week after the organisms became undetectable by PCR, the OF returned to reference values.

Phylogenetic analysis of the 16S rRNA gene of the novel hemoplasma isolate surprisingly revealed a close relationship to M. coccoides, a hemoplasma species isolated from rodents. M. coccoides is known to induce hemolytic anemia in mice and rats (7, 18, 34). M. coccoides has been shown to be mechanically transmitted between mice through the mouseouse Polyplax serrata (2). Experimental transmission of feline hemoplasma species between cats by oral inoculation of infected blood has been successful (9). In view of the permanent outdoor access and successful mousing reported for cat 946, one could speculate that if this new hemoplasma isolate is present in wild rodents in Switzerland, an interspecies transmission from mouse to cat could have taken place through hunting.

So far, 3 out of 250 feline blood samples from Swiss pet cats have tested positive for the newly described hemoplasma isolate using the specific real-time PCR assay (B. Willi, unpublished data). These preliminary data indicate that this agent is of general relevance. In none of these infected cats have characteristic hemoplasma-like inclusions been doubtlessly identified on the blood smears. This could be due to the rather low hemoplasma loads measured in all of the assayed blood samples, compared to the loads previously reported in hemoplasma-infected cats with conclusively positive blood smears (31). The authors therefore recommend that the herein-described real-time PCR assay be used to accurately diagnose infections with the new hemoplasma isolate in felids.

ACKNOWLEDGMENTS

We thank M. Wittenbrink and L. Hoelzle (Vetsuisse Faculty, University of Zurich, Switzerland) for helpful discussion; B. Wenger, U. M. Dreher, C. Brunner, M. Kummerm, C. Baumgartner, V. Fornera, and M. Rios for expert technical help with the experimentally infected cats; and E. Gönczi, H. A. Knorr, B. Weibel, T. Meili Prodan, R. Tandon, E. Rogg, E. Rihner, Y. Bosshart, C. Brönnmann, U. Egger, E. Grässli, M. Huder, B. Lange, M. Nussbaumer, and J. Wächti for excellent laboratory assistance. Laboratory work was performed using the logistics of the Center for Clinical Studies at the Vetsuisse Faculty of the University of Zurich.

This work was supported by a research grant (Forschungskredit 2002) of the University of Zurich, Zurich, Switzerland, and by Merial GmbH, Germany. R.H.-L. is the recipient of a professorship by the Swiss National Science Foundation (PP00B-102860).

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