Use of a polymerase chain reaction assay to detect and differentiate two strains of *Haemobartonella felis* in naturally infected cats

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**Objective**—To develop a polymerase chain reaction (PCR) assay that detects and differentiates the Ohio strain of *Haemobartonella felis* (*H. felis-OH*) and the California strain of *H. felis* (*H. felis-CA*) and to apply the assay to blood samples from cats with and without suspected haemobartonellosis (suspect and control cats, respectively).

**Sample Population**—220 blood samples were examined; 82 were from suspect cats, and 138 were from control cats.

**Procedure**—A PCR assay was designed to detect and differentiate *H. felis-OH* and *H. felis-CA*.

**Results**—On the basis of PCR assay results, the overall prevalence of *H. felis* infection was 19.5% (43/220). Suspect cats (28.0%; 23/82) were significantly more likely than control cats (14.5%; 20/138) to be *H. felis* infected. Significantly greater numbers of suspect cats were *H. felis-OH* infected (12.2%, 9/82) or *H. felis-CA* infected (9.9%, 4/42) than control cats (0.0% [0/138] and 0.5% [1/138], respectively). Significantly more anemic cats were *H. felis-OH* infected (14.3%; 4/28) or *H. felis-CA* infected (9.6%; 3/32) than nonanemic cats (3.4% [2/59] and 1.0% [1/105], respectively). The PCR assay was more accurate than cytologic examination for detection of *H. felis*.

**Conclusions and Clinical Relevance**—*Haemobartonella felis* infections are more common in cats than previously recognized. *Haemobartonella felis-OH* is apparently more pathogenic than *H. felis-CA*. The PCR assay is more accurate than cytologic examination for detection of *H. felis*.

*Haemobartonella felis* is a gram-negative epicellular parasite of feline erythrocytes. The organism was classified as a rickettsia, but recently it has been determined that it is more closely related to mycoplasmal organisms. On the basis of morphologic and genetic characterization, there are at least 2 strains. The small variant of the organism has been designated the California strain of *H. felis* (*H. felis-CA*), and the large form has been designated the Ohio strain of *H. felis* subspecies (*H. felis-OH*). *Haemobartonella felis-OH* is most closely related to *H. muris* whereas *H. felis-CA* is most closely related to *Eperythrozoon suis*.

In cats experimentally inoculated with a *H. felis-OH* isolate, clinical signs consistent with developing anemia were most apparent approximately 3 weeks after inoculation. Recurrence of anemia and clinical disease developed spontaneously in 4 of 11 cats experimentally infected with *H. felis-OH*. Experimental infection with a *H. felis-CA* isolate failed to induce clinical manifestations of disease. In these studies, cytologic detection of the organism was variable after the first 3 weeks of infection and, thus, was less accurate than polymerase chain reaction (PCR) assay for detection of the organism.

In naturally exposed cats, clinical signs and physical examination abnormalities associated with anemia are most common. Although naturally occurring haemobartonellosis is often severe in cats with concurrent FeLV infection, FeLV-naive cats also often develop clinical disease. Fever is commonly present in acutely infected cats and may be intermittent in chronically infected cats. Cats in the chronic phase are usually subclinically infected but can have recurrence of clinical disease following periods of stress. However, recurrence of clinical illness or parasitemia as detected by cytologic examination of blood samples has been difficult to induce experimentally. *Haemobartonella felis* can be detected on the surface of RBC from naturally infected cats but also can be missed on cytologic examination of RBC from cats with clinical haemobartonellosis. It is possible that variation in clinical and laboratory findings in naturally exposed cats may relate to the *H. felis* strain involved. To our knowledge, no one has reported on which *H. felis* strain is detected in naturally exposed cats or attempted to correlate these findings with clinical variables. Objectives of the study reported here were to develop a PCR assay for detection of the currently recognized *H. felis* strains and to apply the assay to blood samples from client-owned cats with and without clinical signs of haemobartonellosis.

**Materials and Methods**

*Haemobartonella felis* 16S rRNA gene sequence analysis—The 16S rRNA gene sequences for *H. felis-OH* (GenBank accessions #U95297 and #U88563), *H. felis-CA* (GenBank accessions #U95298 and #U88564), and the large and small subunit 16S rRNA genes of *Eperythrozoon suis* (GenBank accessions #D89363 and #D89364) were compared by use of the ExPASy server program (http://expasy.org/sprot/)

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accession #U88564), H muriis (GenBank accession #U82963), Mycoplasma felis (GenBank accession #U09787), Bartonella henselae (GenBank accession #73229), Bartonella claridgeiae (GenBank accession #X097822), Escherichia coli (GenBank accession #J01859), Klebsiella pneumoniae (GenBank accession #U33121), and Salmonella enteritidis (GenBank accession #U90318) were aligned by use of a DNA analysis computer program. The 16S rRNA gene sequences for one of the H felis-OH isolates (GenBank accession #U95297) and H muriis (GenBank accession #U82963) are identical (data not shown). Therefore, the sequence for H felis-OH used in our study was derived from GenBank accession #U95297.

**Bacterial strains**—Multiple bacteria were acquired to determine the accuracy of the PCR assay used in our study. Initial isolates of the H felis-OH were donated. An isolate of H felis-CA was obtained from a client-owned, FeLV and FIV seronegative cat examined by one of the investigators (MRL). Other bacteria obtained from American Type Culture Collection included Bartonella henselae (ATCC #49882), Bartonella clarridgeiae (ATCC #51734), Clostridium perfringens (ATCC #3624), Enterobacter cloacae (ATCC #91411), Escherichia coli (ATCC #11755), Fusobacterium necrophorum (ATCC #25286), Klebsiella pneumoniae (ATCC #53555), Pseudomonas aeruginosa (ATCC #51447), Salmonella enteritidis (ATCC #13076), Staphylococcus intermedius (ATCC #10902), Staphylococcus pseudintermedius (ATCC #12393), and Streptococcus spp (ATCC #12277).

**Feline blood samples**—Veterinarians in different regions of the country were contacted by phone and letter to solicit participation in our study. Blood samples were collected from client-owned cats, placed into EDTA, and transported to the laboratory by overnight courier with a cold pack for testing by PCR assay. Samples from cats with suspected haemobartonellosis (suspect cats) on the basis of fever, anemia, or cytologic evidence of infection were submitted. Samples from cats without clinical signs of haemobartonellosis (control cats) were submitted along with suspect samples or were selected from samples for which a CBC was performed at the laboratory for other reasons.

**DNA extraction and PCR amplification of the 16S rRNA gene**—For PCR amplification, DNA was prepared from pure cultures of each bacterial strain or feline blood samples (200 µl) stored in EDTA, using commercially available kits. The PCR amplifications were performed in 50 µl that contained 10 mM Tris, pH 8.3, 50 mM KCl, 3.5 mM MgCl2, 200 µM each dATP, dCTP, and dGTP, 400 µM dUTP, 1 µM each primer, and 2.5 units Taq polymerase. Amplification buffer was optimized with dUTP for use with uracil glycosylase to prevent PCR amplification product carryover. Optimum primer annealing temperatures were determined in an automated gradient temperature cycler. Amplifications were performed in an automated thermal cycler, using a time-release PCR assay protocol as follows: 10-minute incubation at 20 C followed by 2 minutes denaturation at 95 C then 45 cycles of 1-minute denaturation at 95 C, 1-minute annealing at 60 C, and 30-second extension at 72 C. The PCR amplification products were identified by ethidium bromide fluorescence after electrophoresis in 3% agarose gels; gels were then photographed. Haemobartonella felis-positive and H felis-negative blood samples were included as controls in each PCR assay.

**CBC and cytologic examination**—Data from CBC were available for 156 cats. For samples from cats in Colorado, the CBC was performed at the laboratory; and thin blood smears were examined for H felis by a technician and one of the authors (WJR). Information on CBC from other cats in our study was solicited from the referring veterinarian. Samples for which an entire CBC was available were assumed to have had cytologic examination for hemoparasites. For 11 samples, thin blood smears were submitted for cytologic examination of RBC, but a CBC was not available. Because of variation in laboratories and reporting methods, only the PCV or Hct and cytologic detection of H felis were assessed in our study. Cats with a PCV or Hct ≥ 25 were considered to have values within reference range; cats with PCV or Hct < 25 were considered anemic.

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Figure 1—Nucleotide sequence alignment of a portion of the 16S rRNA gene of the Ohio strain of Haemobartonella felis (H felis-OH, GenBank accession #U95297), California strain of H felis (H felis-CA, GenBank accession #U88564), H muriis (GenBank accession #U2963), Mycoplasma felis (GenBank accession #U09787), Bartonella claridgeiae (GenBank accession #U33121), and Salmonella enteritidis (GenBank accession #U90318). Corresponding GenBank nucleotide numbers (nt #) are indicated at the beginning and end of the sequences. Arrows designate polymerase chain reaction (PCR) primer positions. Notice that nucleotides in the 3’ region of the H felis-specific PCR primer sequences are not conserved with other potential pathogens. The 16S rRNA gene sequences predict that these primers should amplify products of 193 and 170 bp from H felis-CA and H felis-OH template DNA, respectively.
Results

PCR assay of *Haemobartonella felis* PCR—Approximately 200 nucleotides in the 5' portion of the 16S rRNA gene contained a nonconserved region that was bordered by areas of high homology (Fig 1). The *H felis* strains differ in the nonconserved region as the result of insertion (or deletion) of 24 nucleotides; primers were designed to amplify this region. The forward and reverse primers were 5'-ACGAAAGTCT-GATGGAGCAATA-3' (*H felis*-CA nucleotides 352 to 373) and 5'-ACGCCCAATAAATCCG(A/G)ATAAT-3' (*H felis*-OH nucleotides 544 to 523), respectively. The 3' nucleotides of the *H felis*-specific PCR primer sequences are not conserved with other potential pathogens. The 16S rRNA gene sequences predict that these primers should amplify products of 193 and 170 bp from *H felis*-CA and *H felis*-OH template DNA, respectively.

As predicted, the expected product sizes were amplified from *H felis*-OH and *H felis*-CA template DNA (Fig 2). Sequencing of the 170- and 193-bp PCR amplification products indicated that the products were specific for *H felis*-OH and *H felis*-CA, respectively (data not shown). Annealing of the 3' nucleotide of the PCR primer is critical for extension by Taq polymerase, thus explaining the inability to amplify a PCR product from template DNA derived from other common bloodborne pathogens of cats including *Bartonella henselae* and *Bartonella claridgeiae*. Similarly, amplification of template DNA derived from other potential pathogens such as *Bartonella claridgeiae*, *Bartonella henselae*, *Bartonella vinsonii* (subsp *berkhoffii*), *Bordetella bronchiseptica*, *Clostridium perfringens*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia risticii*, *Enterobacter cloacae*, *Escherichia coli*, *Fusobacterium necrophorum*, *Klebsiella pneumoniae*, *Mycoplasma felis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus intermedius*, and *Streptococcus spp* did not result in product amplification (data not shown).

Detection of *Haemobartonella felis* by PCR assay—The PCR assay was performed on DNA prepared from 220 blood samples; 82 were from suspect cats, and 138 were from control cats. Most (91%; 126/138) of the control cat samples came from cats that resided in Colorado. On the basis of PCR assay results, 10 cats (4.5%; 10/220) were infected with *H felis*-OH, 28 cats (12.7%; 28/220) were infected with *H felis*-CA, and 5 cats (2.3%; 5/220) were infected with *H felis*-OH and *H felis*-CA, resulting in a prevalence of infection with *H felis* of 19.5% (43/220). Overall, suspect cats (28.0%; 23/82) were more likely (P = 0.014) than control cats (14.5%; 20/138) to be *H felis* infected (Table 1). Significantly greater numbers of suspect cats were *H felis*-OH infected (12.2%; 10/82; P < 0.001) or *H felis*-OH and *H felis*-CA infected (4.9%; 4/82; P = 0.046) than control cats (0% [0/138] and 0.7% [1/138], respectively). There was no difference in the prevalence of *H felis*-CA infection between the suspect and control cats.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>% of suspect cats (No.)</th>
<th>% of control cats (No.)</th>
<th>χ²</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H felis</em>-OH</td>
<td>12.2 (10)</td>
<td>0 (0)</td>
<td>17.631</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><em>H felis</em>-CA</td>
<td>11.0 (9)</td>
<td>13.8 (19)</td>
<td>0.361</td>
<td>0.546</td>
</tr>
<tr>
<td><em>H felis</em>-OH and <em>H felis</em>-CA</td>
<td>4.9 (4)</td>
<td>0.7 (1)</td>
<td>3.995</td>
<td>0.046</td>
</tr>
<tr>
<td>Total</td>
<td>28.0 (23)</td>
<td>14.5 (20)</td>
<td>6.011</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*P* values < 0.05 indicate significant differences between suspect and control cats.

| Statistical analysis—On the basis of PCR assay results, cats were defined as *H felis*-OH-infected, *H felis*-CA-infected, *H felis*-OH- and *H felis*-CA-infected, or *H felis* naïve (to both strains). Frequency distributions for each PCR assay-based category was calculated for cats with and without anemia as well as for cats with suspected *H felis* infection and control cats. Results were compared by χ² analysis with significance defined as P < 0.05.
Results of CBC were available for 156 cats; 28 cats were anemic, and 128 had CBC results within the reference range. Of the available CBC results, most were performed at the reference laboratory. On the basis of the overall PCR assay results, a similar number of anemic cats (7.1%; 2/28) and control cats (13.3%; 17/128) were infected with H felis (Table 2). However, significantly more anemic cats were H felis-OH infected (14.3%; 4/28; P = 0.006) or H felis-OH and H felis-CA infected (7.1%; 2/28; P = 0.026) than control cats (2.3% [3/128] and 0.8% [1/128], respectively).

Only 7 suspect cats (13.2%; 7/53) had positive results for H felis on cytologic examination. Each of these 7 cats had positive PCR assay results concurrently (Table 3). Conversely, there were 9 suspect (17%; 9/53) and 17 control (15.2%; 17/112) cats that had positive results on PCR assay but negative results on cytologic examination.

Discussion

On the basis of the results of our study, the PCR assay was analytically specific because it amplified only H felis DNA and not DNA from other bacterial organisms commonly associated with bacteremia in cats. Detection limits of the described PCR assay cannot be defined in terms of number of organisms per ml of blood, because conditions for the in vitro cultivation or accurate enumeration of H felis organisms have not been described. Detection limits of the PCR assay may be further increased by detection of PCR-amplified products by hybridization with radioactive probes rather than binding of ethidium bromide; however, this was not evaluated.

Pathogenesis of the 2 strains of H felis in naturally infected cats is poorly detailed at this time. In our study, anemic cats and cats suspected to have haemobartonellosis were more likely than control cats to be infected with H felis-OH with or without H felis-CA. In contrast, H felis-CA infection was detected in similar percentages of suspect cats, anemic cats, and control cats. These results support those of others on experimentally infected cats demonstrating that the H felis-OH is more pathogenic than the H felis-CA.

The described PCR assay detected H felis in the blood of all 7 cats that had positive results on cytologic examination as well as an additional 26 cats that had negative results on cytologic examination. These results confirm those of others indicating that PCR amplification is superior to cytologic examination for detection of H felis infection. On the basis of these results, PCR assays should be used for epidemiologic studies of haemobartonellosis. In our study, detection of H felis by PCR amplification in 20 of 138 clinically normal cats indicates that positive results do not always correlate to clinical illness. However, H felis-OH was rarely detected in cats without anemia and was detected in significantly more suspect cats than control cats. Compared with the PCR assay, cytologic examination had false-negative results in 9 of 53 of the suspect cats, which included false-negative results for 3 of 28 anemic cats. Thus, PCR assay also appears to be the test of choice for evaluating clinically ill cats for haemobartonellosis. The morphologic forms of H felis can be difficult to discern cytologically in cats, particularly if only small numbers of cells are parasitized. The described PCR assay has the additional benefit of being able to differentiate the H felis strains infecting cats. Because the H felis strains have apparent differences in pathogenicity, the ability to differentiate strains is likely to have clinical significance.

### Table 2—Prevalence of H felis infections as determined by PCR assay in cats with (n = 28) and without (28) anemia

<table>
<thead>
<tr>
<th>Organisms</th>
<th>% of cats with anemia (%)</th>
<th>% of cats without anemia (%)</th>
<th>χ²</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H felis-OH only</td>
<td>14.3 (4)</td>
<td>2.3 (3)</td>
<td>7.645</td>
<td>0.006</td>
</tr>
<tr>
<td>H felis-CA only</td>
<td>7.1 (2)</td>
<td>13.3 (17)</td>
<td>0.809</td>
<td>0.368</td>
</tr>
<tr>
<td>H felis-OH and H felis-CA</td>
<td>7.1 (2)</td>
<td>0.8 (1)</td>
<td>4.93</td>
<td>0.026</td>
</tr>
<tr>
<td><strong>Total H felis-OH</strong></td>
<td><strong>21.4 (6)</strong></td>
<td><strong>3.1 (4)</strong></td>
<td><strong>12.83</strong></td>
<td><strong>&lt; 0.001</strong></td>
</tr>
<tr>
<td><strong>Total H felis-CA</strong></td>
<td><strong>14.3 (4)</strong></td>
<td><strong>14.1 (18)</strong></td>
<td><strong>0.001</strong></td>
<td><strong>0.976</strong></td>
</tr>
</tbody>
</table>

*Anemia = PCV or Hct < 25%.

See Table 1 for key.

### Table 3—Comparison of the percentage of positive results for H felis by PCR assay and cytologic examination of blood samples from suspect (n = 53) and control (12) cats

<table>
<thead>
<tr>
<th>Results (CE, PCR assay)</th>
<th>% of suspect cats (No.)</th>
<th>% of control cats (No.)</th>
<th>% of all cats (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+) CE; (+) H felis-OH</td>
<td>9.4 (5)</td>
<td>0 (0)</td>
<td>3.0 (5)</td>
</tr>
<tr>
<td>(+) CE; (+) H felis-CA</td>
<td>1.9 (1)</td>
<td>0 (0)</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>(+) CE; (+) H felis-OH</td>
<td>1.9 (1)</td>
<td>0 (0)</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>(+) CE; (+) any PCR</td>
<td>13.2 (7)</td>
<td>0 (0)</td>
<td>4.2 (7)</td>
</tr>
<tr>
<td>(+) CE; (+) H felis-CA</td>
<td>7.5 (4)</td>
<td>0 (0)</td>
<td>2.4 (4)</td>
</tr>
<tr>
<td>(+) CE; (+) H felis-OH</td>
<td>7.5 (3)</td>
<td>14.3 (16)</td>
<td>11.5 (19)</td>
</tr>
<tr>
<td>(+) CE; (+) H felis-OH</td>
<td>3.8 (2)</td>
<td>0.9 (1)</td>
<td>1.8 (3)</td>
</tr>
<tr>
<td>(+) CE; (+) any PCR</td>
<td>17.0 (9)</td>
<td>15.2 (17)</td>
<td>15.8 (26)</td>
</tr>
<tr>
<td>(+) CE; (+) all PCR</td>
<td>69.8 (37)</td>
<td>84.8 (95)</td>
<td>80.0 (132)</td>
</tr>
</tbody>
</table>

CE = Cytologic examination. (+) = Positive result. (−) = Negative result.

See Table 1 for key.
Detection of both strains of *H. felis* in 3 cats of our study indicates that coinfection can develop. It was previously reported that the *H. felis* strains differ by approximately 15% at the 16S rRNA gene region assessed. Thus, it is likely that genetic variation results in sufficient antigenic variation to prevent the induction of cross protection. Serologically, there is substantial antigenic variation between *H. felis*-OH and *H. felis*-CA; antibodies generated by either strain fail to react with the heterogenous strain in immunofluorescent antibody assays. Whether coinfection by the different strains is of clinical importance is currently unknown. However, it is possible that recurrent illness in naturally infected cats may have resulted from a subsequent exposure to a different strain rather than recurrence of disease from the original infection.

As in experimentally inoculated cats, the acute phase of illness from *H. felis* infection is likely to be transient in naturally infected cats. Because the cats in our study were not assessed longitudinally, it is impossible to determine the duration of PCR assay positive results. However, because *H. felis*-CA was more common than *H. felis*-OH in our control cats, it is possible that cats are ultimately more successful in clearing the more pathogenic *H. felis*-OH. Alternatively, more cats may be naturally exposed to *H. felis*-CA than *H. felis*-OH. Our results also document that *H. felis* infection in cats is common. Of the 23 blood samples from suspect cats that had positive PCR-assay results, 10 blood samples were from suspect cats in Colorado or Wyoming. Flea infestations are uncommon in these states, suggesting there may be an alternate mode of transmission.

References


Correction: Norepinephrine kinetics in dogs with experimentally induced renal vascular hypertension

In table 3 of the article “Norepinephrine kinetics in dogs with experimentally induced renal vascular hypertension” (AJVR, Dec 2000, pp 1538), the unit of measure indicated for urinary excretion of sodium, potassium, and chloride is incorrectly listed as mEq/min. The correct unit of measure for all 3 electrolytes is µEq/min.