Differentiation of *Haemobartonella canis* and *Mycoplasma haemofelis* on the basis of comparative analysis of gene sequences

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Objective—To determine whether *Haemobartonella canis* and *Mycoplasma haemofelis* (formerly known as *H felis* [large form]) can be differentiated by use of comparative analysis of gene sequences.

Sample Population—Blood samples obtained from 3 dogs infected with *H canis* and 2 cats infected with *M haemofelis*.

Procedure—The partial 16S rDNA and ribonuclease P RNA (RNase P) genes were amplified, cloned, and sequenced in blood samples obtained from *H canis*-infected dogs and *M haemofelis*-infected cats. The DNA sequences were subjected to comparative analysis.

Results—The 16S rDNA sequences of *H canis* and *M haemofelis* were nearly identical (homology of 99.3 to 99.7%). In contrast, RNase P gene sequences had a lower degree of sequence homology between the 2 organisms (94.3 to 95.5%).

Conclusions and Clinical Relevance—*Haemobartonella canis* and *M haemofelis* are not identical organisms. Molecular differentiation of *H canis* and *M haemofelis* is more clearly evident by use of comparative analysis of RNase P gene sequences than by comparative analysis of 16S rDNA gene sequences. (*Am J Vet Res* 2002;63:1385–1388)

Members of the genus *Haemobartonella* are gram-negative bacteria that cannot be grown in culture. These organisms infect a number of mammalian hosts and can cause hemolytic anemia. Species identification of the genus *Haemobartonella* has been almost exclusively on the basis of their mammalian hosts.[1] Historically, *Haemobartonella* organisms have been classified in the order Rickettsiales on the basis of their epierythrocytic existence and light microscopic morphologic characteristics.[1] However, genetic data that used 16S rRNA gene sequences and electron microscopy findings (ie, lack of a cell wall) have recently resulted in the reclassification of *Haemobartonella felis* (large form) and *H muris* as *Mycoplasma haemofelis* and *M haemomuris*, respectively.[2,3] Additionally, on the basis of clinical and genetic data, 2 distinct types of epierythrocytic gram-negative bacteria that cannot be grown in culture have been found to infect cats (*M haemofelis* and *H felis*).[4-7] There are also provisional proposals under review to reclassify *H canis* and *H felis* (small form) as *M haemocanis* and *M haemominutum*, respectively.[8,9]

To our knowledge, little research has been done to genetically characterize *H canis*, the *Haemobartonella* agent that infects dogs. Only a single *H canis* 16S rDNA sequence from a single isolate has been reported.[10] That gene sequence was nearly identical to the sequence for *M haemofelis*, and it has been hypothesized that *M haemofelis* and *H canis* may actually be the same organism infecting different species of animals.[11]

Although 16S rDNA usually has the necessary criteria for use in rigorous phylogenetic studies among most microorganisms,[12,13] there are situations in which the 16S rDNA sequences from different microorganisms are nearly identical, and differentiation of these microorganisms at the species level is not possible.[14,15] This appears to be the case for *H canis* and *M haemofelis*.[10,11] When analysis of the 16S rDNA sequences are inconclusive because of a high degree of sequence similarity, analysis of the RNA subunit of **ribonuclease P (RNase P)** may lead to better differentiation of closely related organisms.[16] Ribonuclease P is a ribozyme that removes the 5' leader sequences from tRNA precursors during processing of tRNA. Similar to rRNA, the RNA subunit of RNase P is not translated into protein, and the gene sequence dictates the structure and function of the molecule. In contrast to rRNA, RNase P RNA evolves with a faster molecular clock. This means that RNase P genes acquire more mutations or changes during a shorter period. These changes generally result in a greater degree of sequence dissimilarity in closely related organisms, which can be used to improve our ability to differentiate them, as has been documented for *Chlamydia* organisms.[17,18] The purpose of the study reported here was to isolate and sequence partial 16S rDNA and RNase P RNA genes (*rnpB*) from *H canis* and *M haemofelis* and to determine whether these 2 organisms can be differentiated on the basis of comparative analysis of the 16S rDNA or *rnpB* gene sequences.

Materials and Methods

Sample population—Blood samples from dogs and cats presumed to be infected with *H canis* or *M haemofelis*, as determined on the basis of microscopic examination, were obtained from 5 veterinary medical teaching facilities and 1 local diagnostic laboratory. Four blood samples from dogs infected with *H canis* were obtained (2 from Florida [isolate Red and isolate Barney], 1 from North Carolina, and 1 from Oklahoma). Two blood samples were obtained from cats infected with *M haemofelis*; 1 sample was from North

Received Dec 17, 2001.
Accepted May 15, 2002.
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Carolina, and 1 sample was from Illinois.[a] All blood samples contained EDTA as an anticoagulant.

Procedure—Total DNA was extracted from 200 µL of EDTA-anticoagulated blood by use of a commercially available kit[b] performed in accordance with the manufacturer's instructions. Total DNA that had been extracted from a healthy **specific-pathogen-free (SPF)** dog and a healthy SPF cat for use in other studies were used as templates for the **polymerase chain reaction (PCR)** assay in the study reported here.

Partial 16S rDNA was amplified by use of primers 8F and 1492R, which amplify nearly the entire length of 16S rDNA from most prokaryotes.[19] Each 50-µL reaction contained 1X reaction buffer,[c] 1.25 units of *Taq* polymerase,[d] 200 µM of each **deoxynucleoside triphosphate (dNTP)**, 200 ng of genomic DNA, and 50 pmol of each primer (ie, 8F and 1492R). Cycling conditions were 95°C for 10 minutes, which was followed by 35 cycles (95°C for 1 minute, 48°C for 1 minute, and 72°C for 2 minutes) and a final extension step at 72°C for 5 minutes. Cycling was accomplished in a thermal cycler.[e]

Fragments of the RNase P gene were amplified by use of primers designed by the authors to amplify partial *rnpB* from the family Mycoplasmataceae. The primers used were 80F1 (5'–GAG GAA AGT CCR YGC TWG CAC–3' [where R = A or G, Y = C or T, and W = A or T]) and 290R1 (5'–TCC CYT ACC RAA ATT TRG GTT TCT–3' [where R = A or G and Y = C or T]). Each 50-µL reaction contained 1X reaction buffer,[c] 1.25 units of *Taq* polymerase,[d] 200 ng of genomic DNA, 1.5mM $MgCl_2$, 15 nmoles of each primer (ie, 80F1 and 290R1), and 200µM of each dNTP. The PCR conditions were 95°C for 5 minutes, which was followed by 35 cycles (95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute) and a final extension step at 72°C for 5 minutes. Cycling was accomplished in a thermal cycler.[e]

The PCR products were cloned into a plasmid vector,[f] and an *Escherichia coli*[g] strain was transformed in accordance with the protocol of the supplier. Recombinants were selected on the basis of blue-white color of colonies. Plasmid DNA from at least 3 clones for each isolate was isolated.[h] Recombinant plasmid DNA was sequenced bidirectionally by use of infrared fluorescent-labeled primers M13R-700 (5'–CAG GAA ACA GCT ATG ACC ATG–3') and T7-800 (5'–TAA TAC GAC TCA CTA TAG GGC GA–3'). Internal sequencing primers (515F and 1391R) were used for 16S rDNA sequencing.[19] Sequencing reaction conditions were 2 minutes at 92°C followed by 30 amplification cycles (30 seconds at 92°C, 15 seconds at 55°C, and 30 seconds at 72°C).[e] Products of sequencing reactions were analyzed by use of polyacrylamide gel electrophoresis (3.75% gels) on an automated DNA sequencer.[i] Each 16S rDNA sequence was aligned with the published 16S rDNA sequences for *H canis* (GenBank accession No. AF197337), *M haemofelis* (GenBank accession No. AF178677, U88563, and U95297), and *M haemomuris* (GenBank accession No. U82963) by use of 1,357 homologous nucleotide positions.[j] The *rnpB* sequences, excluding the primers, were aligned accordingly by use of 176 nucleotide positions.[j] Sequence differences were evaluated on the basis of corresponding 16S rRNA and RNase P RNA secondary structures. The DNA sequences used for comparison in this study were *M haemofelis* North Carolina 16S rDNA (GenBank accession No. AY069948), *H canis* North Carolina 16S rDNA (GenBank accession No. AF407208), *H canis* North Carolina *rnpB* (GenBank accession No. AF407209), *M haemofelis* North Carolina *rnpB* (GenBank accession No. AF407210), *H canis* Florida Barney *rnpB* (GenBank accession No. AF407211), *M haemofelis* Ohio *rnpB* (GenBank accession No. AF407212), and *H canis* Florida Red *rnpB* (GenBank accession No. AF407213).

Results

A product of approximately 1,450 base pairs was amplified, cloned, and sequenced from isolates cultured from blood samples obtained from infected dogs and cats, except for the blood sample from the dog from Oklahoma. Amplicons were not detected in the samples from the SPF dog or cat as well as the control samples that did not contain DNA. The *H canis* 16S rDNA sequences from North Carolina and Florida were identical. The 16S rDNA sequences from the *M haemofelis* samples from North Carolina and Illinois also were identical.

The 16S rDNA sequences from *H canis* and *M haemofelis* were compared with sequences in the GenBank database and with each other by use of 1,357 nucleotide positions. All of the *H canis* and *M haemofelis* sequences were nearly identical, with sequence homology of ≥ 99%. Interestingly, the *H canis* 16S rDNA sequences determined in this study were more similar to the *M haemofelis* 16S rDNA sequences AF178677 and U88563 than was the *M haemofelis* sequence U95297 **(Table 1)**.

A product of approximately 220 base pairs was amplified, cloned, and sequenced from isolates cultured from blood samples obtained from infected dogs and cats, except for the blood sample from the dog from Oklahoma. Amplicons were not detected in samples from the SPF dog or cat as well as the control samples that did not contain DNA. The *H canis rnpB* sequences from Florida and North Carolina had a high degree of homology (97.7 to 100%). The *M haemofelis rnpB*

Table 1—Comparison of the *Haemobartonella and Mycoplasma* 16S rDNA sequences determined in the study reported here with other reported *Haemobartonella* and *Mycoplasma* 16S rDNA sequences by use of a multiple-sequence alignment program that used 1,357 nucleotide positions

No.	Isolate (GenBank Accession No.)	1	2	3	4	5	6	7
1	*H canis* (AF407208)*	—	100	99.6	99.7	99.7	99.3	87.2
2	*H canis* (AF197337)	100	—	99.6	99.7	99.7	99.3	87.2
3	*M haemofelis* (AY069948)†	99.6	99.6	—	99.8	99.8	99.6	87.0
4	*M haemofelis* (AF178677)	99.7	99.7	99.8	—	100	99.5	87.0
5	*M haemofelis* (U88563)	99.7	99.7	99.8	100	—	99.5	87.0
6	*M haemofelis* (U95297)	99.3	99.3	99.6	99.5	99.5	—	86.7
7	*M haemomuris* (U82963)	87.2	87.2	87.0	87.0	87.0	86.7	—

*Sequence is representative of the *H canis* 16S rDNA sequences obtained from the North Carolina, Florida Red, and Florida Barney isolates. †Sequence is representative of the *M haemofelis* 16S rDNA sequences obtained from the North Carolina and Illinois isolates.
— = Not applicable.

Table 2—Comparison of the *Haemobartonella* and *Mycoplasma* ribonuclease P sequences determined in the study reported here by use of a multiple-sequence alignment program that used 176 nucleotide positions.

No.	Isolate (GenBank Accession No.)	1	2	3	4	5
1	*H canis* North Carolina (AF407209)	—	100	97.7	95.5	94.9
2	*H canis* Florida Red (AF407213)	100	—	97.7	95.5	94.9
3	*H canis* Florida Barney (AF407211)	97.7	97.7	—	95.5	94.3
4	*M haemofelis* North Carolina (AF407210)	95.5	95.5	95.5	—	99.4
5	*M haemofelis* Illinois (AF407407212)	94.9	94.9	94.3	99.4	—

— = Not applicable.

sequences from Illinois and North Carolina were nearly identical (99.4%). However, in contrast to the 16S rDNA sequences, there was a greater degree of sequence dissimilarity between *H canis* and *M haemofelis rnpB* sequences (94.3 to 95.5%; **Table 2**). These differences in *rnpB* sequences should enable investigators to better differentiate between *H canis* and *M haemofelis*.

The partial RNase P RNA genes, excluding the primer sequences, from *M haemofelis* (176 nucleotides) and *H canis* (177 nucleotides) resembled the nucleotides at positions 63 to 240 of the *Bacillus subtilis* (B-type) RNase P RNA. On the basis of size of *B subtilis* RNA (401 nucleotides), our PCR fragments (excluding the primer sequences) represented approximately 44% of the probable size for the investigated RNase P RNA. The corresponding structures conformed to the homologous regions of P5 to P12 and P15.1 of the general B-type RNase P RNA.

Discussion

Although comparative analysis of 16S rDNA sequences is considered to be one of the most powerful tools for use in determining the phylogenetic affiliation among microorganisms, it does not always provide differentiation for microorganisms at or above the species level. Results of the study reported here and another study[10,11] have revealed that *H canis* 16S rDNA sequences are nearly identical to *M haemofelis* 16S rDNA sequences; thus, it is concluded that the minor nucleotide differences in the 16S rDNA sequences make it nearly impossible to differentiate *H canis* and *M haemofelis*.

The aforementioned results raised the possibility that *H canis* and *M haemofelis* may actually be identical organisms with the ability to infect multiple hosts.[11] To further investigate this possibility, we evaluated a second phylogenetically informative gene (ie, *rnpB*). We found that *H canis* and *M haemofelis rnpB* genes were not identical and that they had a greater degree of sequence dissimilarity than the 16S rRNA genes. Results of comparative analysis of gene sequences for *rnpB* genes did not support the hypothesis that *H canis* and *M haemofelis* are identical organisms. However, in our opinion, these data alone are insufficient to determine whether *H canis* and *M haemofelis* should be classified as different species, subspecies, or strains of a larger (ie, newly named) species. Despite these findings, it is likely that *H canis* will be reclassified as *Mycoplasma haemocanis*.[8]

The *H canis* Florida Barney *rnpB* sequence had some degree of sequence dissimilarity when compared with the other 2 *H canis* isolates, despite having an identical 16S rDNA sequence. Although results of the *rnpB* sequence analysis still support differentiation of the *H canis* and *M haemofelis* isolates, the sequence variation between *H canis* strains may mean that this region of the gene is not suitable for the development of discriminatory diagnostic tests that do not require DNA sequencing. We surmise that *H canis* and *M haemofelis* are not genetically identical organisms, but further characterizations such as DNA-DNA hybridization and sequence analysis of other genes and more isolates will be needed to define their final taxonomic position.

[a]Dr. Joanne Messick, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL 61802, generoulsy provided *H felis* (large form) DNA.
[b]Qiagen Blood Mini Kit, Qiagen Inc, Valencia, Calif.
[c]Geneamp PCR Buffer II, Applied Biosystems, Foster City, Calif.
[d]Amplitaq Gold, Applied Biosystems, Foster City, Calif.
[e]PCR Express, Hybaid Limited, Middlesex, UK.
[f]PCR 2.1, Invitrogen, Carlsbad, Calif.
[g]TOP10', Invitrogen, Carlsbad, Calif.
[h]QIAprep Spin Miniprep Kit, Qiagen Inc, Valencia, Calif.
[i]LI-COR 4200 DNA Sequencer, LI-COR Inc, Lincoln, Neb.
[j]MegAlign, DNAStar Inc, Madison, Wis.

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