

Coinfection with Three *Ehrlichia* Species in Dogs from Thailand and Venezuela with Emphasis on Consideration of 16S Ribosomal DNA Secondary Structure

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As part of a larger study to investigate tick-borne infections in dogs from Thailand and Venezuela, documentation of coinfection with three *Ehrlichia* species in two dogs, one from each country, became the focus of the present study. Although neither dog had clinical signs attributable to ehrlichiosis, both dogs were anemic and neutropenic and the Thai dog was thrombocytopenic. Genus- and species-specific PCR targeting the 16S rRNA genes indicated that both dogs were coinfecting with *Ehrlichia canis*, *E. platys*, and *E. equi*. To our knowledge, these results provide the first molecular documentation for the presence of *E. equi* in dogs from these countries. Using universal bacterial PCR primers, one nearly full-length 16S rRNA gene could be amplified from each dog. The sequences were identical to each other and almost identical to that of *E. platys* (AF156784), providing the first *E. platys* 16S ribosomal DNA (rDNA) sequences reported from these two geographically divergent countries. To determine whether these sequence differences allow differentiation between these two strains and other published 16S rDNA *E. platys* sequences, we performed a phylogenetic analysis of the rRNA, incorporating the consideration of secondary structure.

Dogs can be infected with several *Ehrlichia* species, including *Ehrlichia canis* (7), *E. chaffeensis* (6), *E. equi* (17), *E. risticii* (15), *E. platys* (12), and *E. ewingii* (9). Knowledge related to the geographic distribution, zoonotic potential, and pathologic consequences of *Ehrlichia* infections in dogs has expanded in recent years. However, within the genus *Ehrlichia*, only *E. canis* has been strongly implicated as a canine pathogen of worldwide distribution. In Thailand, morulae have been visualized in canine monocytes and platelets, whereas in Venezuela, morulae have been observed in monocytes, granulocytes, and platelets (1). With the advent of increased serologic and molecular testing, coinfection with multiple tick-borne organisms has been recognized with increasing frequency in both dogs and humans in the United States (2, 4, 9, 16, 18). Data related to coinfection with multiple tick-borne pathogens are less available from many other countries.

Cultivation of *Ehrlichia* sp. requires complex and time-consuming steps, large blood specimen volumes, and meticulous attention to detail. Additionally, the phenotypic characterization of intracellular bacteria can lead to the proposal of a novel organism that genotypically may or may not be different from other known organisms. Phylogenetic analysis of 16S rRNA has proven to be the most powerful tool for the identification and classification of microorganisms (20, 23) and does not rely on the cultivation of organisms. Therefore, it has become the approach of choice when phenotypic data are inconclusive. In this report, we have utilized this approach to identify and to

characterize the different *Ehrlichia* species responsible for coinfection in the dogs from Thailand and from Venezuela.

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MATERIALS AND METHODS

Dogs. The dog from Thailand (a 6-year-old female poodle) was admitted to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand, for evaluation of periodontal disease. Blood from the dog from Venezuela (an adult male mixed-breed dog) was sent to Unidad de Investigaciones Clínicas, Facultad de Ciencias, Veterinarias, Universidad del Zulia, Maracaibo, Venezuela, for hematologic evaluation. The dog was reportedly healthy, but another dog from the same household had died recently of a febrile illness compatible with ehrlichiosis, raising the possibility for a tick-transmitted infection. Neither dog had traveled outside of the country of origin.

Blood sample collection. Half of the blood obtained from the dog from Thailand was treated with EDTA as an anticoagulant, and the remainder was allowed to clot for the removal of serum. For the dog from Venezuela, only EDTA-anticoagulated blood was available to us. Samples were stored frozen at -70°C and transported on dry ice.

IFA and Western immunoblotting. An indirect fluorescent antibody (IFA) test was performed on the serum from the Thai dog to assess the prevalence of antibodies to *E. canis*, *E. chaffeensis*, *E. equi*, and *E. risticii* (14). To confirm the IFA results, serum from the Thai dog was screened by electrophoretic analysis of *E. canis* (canine-origin strain Florida, provided by C. J. Holland) and *E. phagocytophila* (human-origin strain 96HE158, provided by J. S. Dumler) protein antigens using the Western immunoblotting procedure, as described elsewhere (21).

DNA extraction and PCR amplification. Frozen (-70°C) EDTA-blood was thawed to room temperature, and 200 μl was removed and washed twice with phosphate-buffered saline. DNA was extracted using the QIAamp DNA-blood minikit (Qiagen, Chatsworth, Calif.) by following the manufacturer's protocol. To minimize the potential risks for contaminations, DNA extractions, PCRs, and agarose gel electrophoresis were performed in separate rooms. Positive (tissue culture-grown *Ehrlichia* species) and negative controls were included in all PCR assays.

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PCRs for the amplification of partial 16S ribosomal DNA (rDNA) for the genus *Ehrlichia* (122 bp) and for *Ehrlichia* species (151 to 401 bp, depending on the species) were performed by using nested PCR in a Progene thermocycler (Technique, Princeton, N.J.) as previously described (4, 16). PCR amplification of the almost-complete 16S rDNA (1,460 bp) was accomplished with primers PO-C and PC-5A as previously described (22), except that the annealing temperature was 53°C. All PCR products were separated by agarose gel electrophoresis in 1× Tris-borate-EDTA buffer. The concentration of agarose was 1 (386 to 1,460 bp) or 2% (122 to 151 bp). PCR products were purified by using the Qiaquick PCR purification kit (Qiagen) as described by the supplier. The DNA fragments were visualized by UV transillumination after ethidium bromide staining and compared to DNA size standards (Promega, Madison, Wis.).

Cloning and sequencing. PCR amplicons that represented almost-full-length 16S rDNA (1,460 bp) were ligated into the pCR 2.1-TOPO vector followed by transformation of *Escherichia coli* TOP 10 cells using the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. The resulting clones were screened by blue/white colony screening. Plasmid DNA of positive clones was isolated by using the QIAprep spin miniprep kit (Qiagen). Size confirmation of the cloned inserts was performed by restriction digest with *EcoRI* and subsequent agarose gel electrophoresis. Each insert of interest was reamplified from the plasmid using plasmid-specific primers M13 forward (–20) and M13 reverse. The resulting amplicons were digested with restriction enzyme endonucleases *AluI* and *HpaI* (Promega). DNA fragments were separated on a 4% agarose gel in 1× Tris-borate-EDTA. Clones that represented unique restriction fragment patterns were chosen for double-strand sequencing. All sequencing reactions were performed at the Center Sequencing Facility (University of North Carolina, Chapel Hill, N.C.). The following primers were used: P1 (5'ACTCTACTCGGGAGGCAGCAGT), P3Mod (5'ATTAGATACCCTGGTAGTCC), and P4 (5'GAGGAAGGTGGGGACGTCAA) and M13 reverse (Invitrogen), PC1 (5'ACTGCTGCCTCCCGTAGGAGT), PC3 (5'GGACTACCA GGGTATCTAAT), and PC4 (5'TTGACGTCATCCCCACCTTCCTC). Short PCR products derived from the species-specific PCRs were used directly for sequencing with primers HE3-R (5'CTTCTATAGGTACCGTCATTATCTTC CCTAT) and EC-F (5'CAATTATTTATAGCTCTGGCTATAGGAA) for *E. canis*, HE3-R and EQ-F (5'GTGCAACGGATTATCTTTATAGCTTGC) for *E. equi*, and GE2f (5'GTTAGTGGCAGACGGGTGAGT) and Ehr13-IP2 (5'T CATCTAATAGCGATAAATC) for *E. platys*.

Sequence analysis. All 16S rDNA sequences were initially compared to the sequence data available in the common databases by using BLAST, version 2.0 (3) for the determination of their approximate phylogenetic affiliations. Sequences that reflected the closest matches (those for *E. canis* [U26740], *E. platys* [AF156784], *E. equi* [M73223], *E. phagocytophila* [M73220], human granulocytic ehrlichiosis (HGE) agent [AF093788]) were downloaded from GenBank and were aligned with the sequences from this study based on 16S rRNA secondary structure. Sequence differences were evaluated based on the comparison of homologous regions within the 16S rRNA.

Nucleotide sequence accession numbers. Partial 16S rDNA sequences derived from Thai and Venezuelan dogs were deposited in GenBank under accession no. AF287155 and AF287154 for *E. equi* and under accession no. AF286699 and AF287153 for *E. platys*.

RESULTS

Case summaries. The dog from Thailand had no signs of abnormal bleeding, was afebrile, and had a systolic heart murmur. During blood smear examination, intracellular morulae (cell type not defined) containing *Ehrlichia* species were observed and *Babesia canis* parasites were identified in erythrocytes. The dog was anemic (18.5% packed cell volume [PCV]; reference values, 37 to 55%), leukopenic (6,100/μl; reference values, 6,900 to 13,600/μl), neutropenic (3,172/μl; reference values, 3,300 to 9,000/μl), and thrombocytopenic (30,000/μl; reference values, 150 to 500/μl). In the dog from Venezuela, morulae containing *Ehrlichia* species were found in granulocytes and in platelets. Additionally, gamonts of the eucaryotic genus *Hepatozoon* were reported in neutrophils. The dog was anemic (18.5% PCV; reference values, 37 to 55%), leukopenic (3,600/μl; reference values, 6,900 to 13,600/μl), neutropenic (2,988/μl; reference values, 3,300 to 9,000/μl), and

lymphopenic (216/μl; reference values, 1,200 to 4,200/μl). Values are given in cells per microliter of blood.

Serology. By IFA testing, the Thai dog had a reciprocal titer of 10,240 to *E. canis* antigens, 10,240 to *E. chaffeensis* antigens, 1,280 to *E. equi* antigens, and 640 to *E. risticii* antigens. The Western immunoblot patterns with respect to *E. canis* and *E. equi* antigens were indicative of the exposure to *E. canis*.

Sequencing and analysis of species-specific PCR products. Partial 16S rRNA sequences obtained from *Ehrlichia* species-specific PCR products provided the initial molecular evidence that both dogs were infected with at least three *Ehrlichia* species. The sequences of the *E. canis* and *E. platys* PCR products from both dogs (302 and 129 bp, respectively) were identical to those of the corresponding regions in the 16S rDNAs of *E. canis* (U26740) and *E. platys* (AF156784), respectively. The 343-bp partial 16S rDNA fragments derived from both dogs that resembled that of *E. equi* were identical to each other but showed differences in three positions from those of *E. equi* (M73223), *E. phagocytophila* (M73220), and HGE agent (AF093788).

Analysis of *E. platys* 16S rDNA. By using universal bacterial primers PO-C and PC-5A (22), we amplified the almost-complete (1,460 bp) 16S rDNA from one of the organisms involved in the coinfection of the two dogs studied. The 16S rDNA sequence derived from the Thai dog differed in five positions from the corresponding sequence for *E. platys* (strain Gzh981; China) deposited in GenBank (AF156784). Three of these differences are at nucleotide positions 1078, 1142, and 1309 of the corresponding rRNA (*E. coli* J01695 numbering system). Two single nucleotide insertions occur between nucleotide positions 511 and 512 and between positions 990 and 991 (*E. coli* J01695 numbering system). The 16S rDNA sequence obtained from the Venezuelan dog differed from *E. platys* 16S rDNA (AF156784) in four positions. Two differences are at nucleotide positions 1078 and 1299, and two single nucleotide insertions are between nucleotide positions 511 and 512 and between positions 990 and 991 of the corresponding rRNA (*E. coli* J01695 numbering system). The 16S rDNAs from both dogs differed from each other in only three positions (1142, 1299, and 1309) of the corresponding rRNAs. The sequence differences will be considered in Discussion.

DISCUSSION

In this study, we focused on two dogs from geographically diverse countries that were coinfecting with *E. canis*, *E. platys*, and *E. equi*. Western immunoblot analysis confirmed exposure to *E. canis* but could not be used to confirm exposure to *E. equi* because of the cross-reaction of *E. canis* serum to *E. equi* antigens (21). Similarly, sera from dogs infected with *E. canis* or *E. chaffeensis* are also highly cross-reactive (5, 16). Since serum was not available from the Venezuelan dog, serologic testing could not be performed.

To overcome the limitations of serology, 16S rDNA-based PCR was used to obtain molecular evidence for coinfection with *E. canis*, *E. platys*, and *E. equi*. Phylogenetic analysis using 16S rRNA is ideally based on the comparison of homologous regions of the 16S rRNA molecules. This has to be accomplished by using an alignment based on secondary structure rather than simply aligning sequences based on sequence sim-

ilarity (20, 23). The main problem one encounters when comparing 16S rRNA sequence data with those derived from common databases is how to evaluate sequence differences and how to derive conclusions about the relatedness of organisms. However, differences can be the result of, e.g., sequencing errors, PCR errors, or microheterogeneity between different rRNA operons within the same organism (10).

By comparing our almost-full-length 16S rDNA sequence data derived from the dogs in Thailand and Venezuela to the *E. platys* sequences deposited in GenBank (3) under accession no. AF156784, we found almost sequence identity. All sequence differences in our data set have been confirmed in independent experiments by the double-strand sequencing of the corresponding DNA. Five and four positions of the 16S rDNA out of a total of 1,429 bp were different between *E. platys* (AF156784) and the *E. platys* sequence derived from the Thai and Venezuelan dogs, respectively.

To evaluate the accuracy and the importance of these sequence differences for phylogenetic studies and the development of specific PCR primers or diagnostic DNA/RNA probes, we performed a phylogenetic analysis based on secondary structure (<http://www.rna.icmb.utexas.edu>). We believe that the insertion of a C between positions 511 and 512 (*E. coli* J01695 numbering system), as reported for *E. platys* (AF156784), is due to a sequencing or PCR artifact. This insertion was not observed in our sequences, and the corresponding positions are conserved within the *Bacteria*. There is no evidence from the secondary structure of the 16S rRNA to support the presence of this insertion. The reported insertion of a T (AF156784) at positions 990 and 991 (*E. coli* J01695 numbering system) is in a loop area and is therefore possible. However, none of our sequencing data show this insertion. *E. platys* (AF156784) shows a deletion at position 1078 (*E. coli* J01695 numbering system). The sequence of the corresponding loop is therefore GGA. Both our isolates show a G at this position, which is part of a tetraloop with sequence GGGA. A tetraloop at this position is widely present in bacterial 16S rRNAs. Furthermore, this tetraloop is of the GNRA type (R = purine), one of the most common motifs in terminal loops within RNA molecules (13). We therefore believe that the deletion in *E. platys* (AF156784) could also be a result of a sequencing or PCR artifact. At position 1142 (*E. coli* J01695 numbering system), we observed A for the sample from Thailand, whereas *E. platys* (AF156784) and the sample from Venezuela have a G. Since this position is part of a conserved helix and since our sequence did not support a covariation event, we believe that an A at position 1142 is highly unlikely to occur in vivo and might be due to a PCR or sequencing error. Position 1299 (*E. coli* J01695 numbering system) is located in a loop that is occupied by an A for *E. platys* (AF156784) and the sequence derived from the Thai dog, whereas the sequence from the Venezuelan dog has a G. We therefore consider this result as possible.

Secondary structures of RNA molecules are based on Watson-Crick and non-Watson-Crick base pairs, e.g., G · U (19). At position 1309, the sequence derived from the Venezuelan dog and the sequence of *E. platys* (AF156784) have a T, whereas the sequence derived from the Thai dog has a C. This position is in a stem structure that has been confirmed by covariation. Since G · U base pairs in RNA stem structures are possible, we consider this result valid.

The results clearly indicate that one of the infecting organisms in both cases is *E. platys*. The minor sequence differences within the 16S rRNA molecules do not allow phylogenetic differentiation between *E. platys* from China, Thailand, and Venezuela (10). Nevertheless, the few differences in the RNA sequence can be used to develop PCR primers or DNA/RNA probes, subject to a determination of the legitimacy of these differences in the 16S rRNA sequences as outlined above.

The *E. equi*-specific PCR primers amplified DNA fragments (401 bp) from both dogs. Due to direct sequencing of the PCR products, only 343 bp was available for the comparison to other sequences deposited in the common databases. Our two sequences were found to be identical to each other. With the exception of three positions, the two sequences were identical to the 16S rRNA data deposited in GenBank for *E. equi* (M73223), HGE agent (AF093788), and *E. phagocytophila* (M73220).

Based on the secondary structure analysis, these three positions are located within a helix that corresponds to positions 61 to 106 (*E. coli* J01695 numbering system). This helix is a common structural feature within the *Bacteria*. However, only portions of the helix (positions 61 to 67 and 101 to 106 and positions 81 to 88) are conserved. The nucleotide positions between those regions, as well as the length of the helix, can vary and are 17, 20, and 21 bp in *E. coli*, *E. equi* from Thailand and Venezuela, and the *E. equi* sequence from GenBank (M73223), respectively. These minor differences are inadequate to distinguish our samples from *E. equi* (M73223), HGE agent (AF093788), and *E. phagocytophila* (M73220).

All the sequence differences identified in this study are inadequate to phylogenetically support the presence of a novel *Ehrlichia* species. Based on 16S rRNA there is so far no meaningful differentiation between the same *Ehrlichia* species from these geographically divergent locations.

To our knowledge, this study represents the first molecular evidence that *E. canis*, *E. platys*, and *E. equi* infect dogs in Thailand and Venezuela. This study further supports the hypothesis that coinfection with multiple *Ehrlichia* species occurs in dogs. The extent to which coinfection potentiates disease manifestations or complicates the diagnostic and therapeutic management of sick dogs awaits the results of future studies.

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