Anaplasma platys in Dogs, Chile

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We conducted a 16S rRNA nested PCR for the genus Ehrlichia and Ehrlichia spp. with blood samples from 30 ill dogs in Chile. Phylogenetic analysis was performed by using groESL gene amplification. We identified Anaplasma platys as 1 of the etiologic agents of canine ehrlichiosis.

Ehrlichioses are recognized as important emerging tick-borne diseases in humans and wild and domestic animals. The brown dog tick, Rhipicephalus sanguineus, is the main tick that infests dogs in Chile (1). This tick species is a vector of Ehrlichia canis and has been implicated, but not confirmed, as a vector of Anaplasma platys (2). Serologic and clinical evidence of canine ehrlichiosis and serologic evidence of human ehrlichiosis have been reported in Chile (3,4). The purpose of this study was to identify the etiologic agent of canine ehrlichiosis in Chile.

The Study
Blood samples were obtained from 30 pet dogs seen in a private veterinary clinic in Santiago, Chile, with tick infestation and clinical signs compatible with ehrlichiosis (hemorrhagic manifestations and thrombocytopenia). We performed a nested PCR to amplify a portion of the 16S rRNA gene by using specific primers for the genus Ehrlichia and for Ehrlichia spp. DNA was extracted from 300 μL of whole blood by using the Wizard Genomic DNA Puriﬁcation kit (Promega, Madison, WI, USA). For Ehrlichia genus–speciﬁc PCR, 2.5 μL of DNA was ampliﬁed by using outer primers EHR-OUT1 and EHR-OUT2 and inner primers GE2F and EHRL3-IP2 in 1 reaction with a ﬁnal volume of 25 μL (5) (Table 1).

The ﬁrst-round ampliﬁcation included 20 cycles of denaturation at 94°C for 45 s, annealing at 72°C for 1.5 min, and chain extension at 72°C for 1.5 min. The second-round ampliﬁcation included 50 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, and chain extension at 72°C for 1 min, followed by a ﬁnal extension at 72°C for 5 min. Ampliﬁcation products were analyzed by agarose gel electrophoresis. The expected size of the ampliﬁcation product was 120 bp. A. phagocytophilum DNA was used as a positive control (provided by Didier Raoult). For Ehrlichia spp.–speciﬁc ampliﬁcation, we used the same set of outer primers for Anaplasmataceae and speciﬁc inner primers for A. phagocytophilum (6), E. chaffeensis, E. ewingii, and E. canis (5) (Table 1). For A. platys ampliﬁcation, we used inner primers developed by Kordick et al. (EHL3-IP2–E. platys) (7) (Table 1). Expected sizes of ampliﬁcation products were 546, 395, 395, 389, and 151 bp, respectively.

The Ehrlichia genus PCR resulted in the expected DNA band in 6 of 30 dogs (dogs 7, 12, 17, 19, 23, and 25). These 6 samples were positive only for A. platys, showing the expected 151-bp product, and negative for other species tested (Figure 1, panel A). A. platys PCR was also conducted on the remaining 24 Ehrlichia-negative samples; none were positive.

DNA obtained from 3 16S rRNA PCR products (dogs 7, 17, and 25) was puriﬁed by using a commercial kit (Rapid Gel Extraction System; Marligen Biosciences, Ljamsville, Germany) and sequenced twice with an ABI 3100 genetic analyzer (Model 3100; Applied Biosystems, Foster City, CA, USA). The 16S rRNA sequences obtained were compared by using BLAST (www.ncbi.nlm.nih.gov/blast) with sequences available at GenBank. Sequences obtained were similar to that of A. platys strain Okinawa 1 (GenBank accession no. AF536828), with similarities of 98%, 95%, and 98%, respectively. GenBank accession nos. for 16S RNA sequences of A. platys strains obtained in this study are DQ125260 and DQ125261, which correspond to strains from dogs 7 and 17, respectively.

For phylogenetic analysis, the groESL gene of A. platys was ampliﬁed from samples positive for A. platys 16S rRNA that had sufﬁcient amounts of DNA (dogs 17, 23, and 25) and from 1 negative sample (dog 13). Reactions contained 2 μL of puriﬁed DNA as template in a total volume of 25 μL. Ampliﬁcations contained 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 3 mmol/L MgCl2, 2.5 mmol/L deoxyribonucleotide triphosphates (Invitrogen), and 0.2 pmol/L of primers EEgro1F and EEgro2R (8) (Table 1). DNA was denatured by heating at 95°C for 10 min. PCR ampliﬁcation included 40 cycles of denaturation at 95°C for 1.5 min, annealing at 52°C for 2 min, and extension at 72°C for 1.5 min, followed by a ﬁnal extension at 72°C for 10 min. For nested ampliﬁcations, 1 μL of primary PCR products was used as the template in a total volume of 25 μL. Reaction conditions were the same as for primary ampliﬁcations. The primers used were SQ3F, SQ5F, SQ4R, and SQ6R (9) (Table 1). PCR products were analyzed by 1.5% agarose gel electrophoresis.

We ampliﬁed 3 overlapping fragments (790, 1,170, and 360 bp) in 3 16S rRNA–positive samples (Figure 1, panel B). These DNAs were puriﬁed by using a commercial kit (Rapid Gel Extraction System; Marligen), sequenced, and analyzed for phylogenetic relationships. Multiple alignment

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A phylogenetic tree was constructed by the neighbor-joining method and distance matrices for the aligned sequences were calculated by using the Kimura 2-parameter method. Stability of the tree was estimated by bootstrap analysis of 1,000 replications.

Calculation of distance matrices and analysis was performed with the ClustalW program (www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed by the neighbor-joining method and distance matrices for the aligned sequences were calculated by using the Kimura 2-parameter method. Stability of the tree was estimated by bootstrap analysis of 1,000 replications. A final sequence of 686 bp obtained from the overlapping fragments was used for comparison and showed 100% identity between the 3 Chilean sequences and 99.8% similarity with sequences of the A. platys group deposited in GenBank. This finding has also been observed in strains from different geographic origins (13).

Conclusions

We identified A. platys DNA in the blood of 6 dogs with clinical signs indicative of ehrlichiosis. These findings support the conclusion that A. platys is an etiologic agent of canine ehrlichiosis in Chile.

Since its first report in the United States in 1978 (10), A. platys has been described in several countries as the etiologic agent of cyclic thrombocytopenia in dogs. A tick vector of A. platys has not been determined, although R. sanguineus is the most suspected species (2). Because R. sanguineus is the only tick species that infests dogs in Santiago (1), our results support the conclusion that this species is the vector of A. platys in Chile.
Recent studies have shown more genetic variability when sequences of the gltA gene were used (11,12).

Evidence of the zoonotic potential of *A. platys* is scarce. In Venezuela, a few symptomatic human cases have been diagnosed since 1992 by the presence of platelet morulae in blood smears (14). Monocytic and platelet morulae were reported in a 17-month-old girl with fever and rash (15). However, none of these cases have been confirmed by molecular assays. Further studies that investigate the pathogenic and zoonotic role of *A. platys* should be conducted.

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