IN VITRO ISOLATION AND MOLECULAR CHARACTERIZATION OF AN EHRlichia canis STRAIN FROM SÃO PAULO, BRAZIL

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ABSTRACT

An Ehrlichia canis isolate was obtained from a naturally infected dog exhibiting clinical signs of ehrlichiosis in São Paulo Municipality, state of São Paulo, Brazil. The isolate was characterized by PCR and DNA sequencing of portions of the ehrlichial genes dsb, 16SrRNA, and p28. Partial dsb and 16S rRNA sequences were identical to three and five other E. canis strains, respectively, from different countries and continents (including North America, Africa, Asia and Europe). Conversely, the p28 partial sequence for this E. canis (São Paulo) differed by 1, 2, and 2 nucleotides from the corresponding sequences of the E. canis strains Jake (from USA), Oklahoma (USA), and VHE (Venezuela), respectively. The results in this study indicate that E. canis is the only recognized Ehrlichia species infecting dogs in Brazil.

Key-words: Ehrlichia canis, Dog, Molecular Characterization, Ehrlichiosis.

INTRODUCTION

Ehrlichia canis is the primary etiologic agent of canine monocytic ehrlichiosis (CME), which is recognized as the most prevalent tick-borne disease affecting dogs in Brazil (1). The widespread distribution of the tick vector (Rhipicephalus sanguineus) in most urban areas of Brazil, combined with a persistent infection of E. canis in dogs, and lack of effective immunity to E. canis, contribute to the high prevalence of CME in the country (1,2,7,14).

CME in Brazil was first described in 1973 by Costa et al. (8). Recent local data suggest that 20-30% of dogs admitted to veterinary hospitals from various areas had antibodies reacting with E. canis antigens and/or had detectable E. canis DNA (6,9,13,21). Despite the widespread distribution of E. canis in Brazil, only two isolates have been propagated in the laboratory: one from Rio de Janeiro city (22) and another from Jaboticabal County, state of São Paulo (2,15). Both isolates were established in a canine monocyte cell line (DH82 cells); however, only one isolate (Jaboticabal) has been molecularly characterized.

E. canis appears to be highly conserved among geographically dispersed isolates. Aguirre et al. (3) described a high identity of 16S rRNA gene sequences in isolates from the United States, Israel, Japan and Venezuela. McBride et al. (17) revealed that a p28 gene from seven different North-American isolates was identical. In addition, partial sequences of the dsb gene of isolates from Cameroon, Brazil, and United States were identical (2,19). In the present study, we report a new isolate of E. canis from a dog in Brazil, which has been established in cell culture. Additionally, we performed a partial molecular characterization of the isolate.

MATERIALS AND METHODS

Case report

A 7-year-old male Labrador retriever was admitted to the São Paulo University Veterinary Hospital with recurrent...
of anorexia, apathy, bloody diarrhea and tick infestation. Based on a previous clinical diagnosis of CME, the dog had been treated with doxycycline (10mg/Kg daily for 3 weeks) and imidocarb dipropionate (5 mg/Kg, two doses with 15 days interval) 20 days before the appearance of clinical symptoms. The dog was normal upon physical examination. Hematological abnormalities were anemia [hematocrit: 33% (reference values: 37 - 55%); low erythrocytes: 4.7 x 10⁸/mm³ (reference values: 5.5 - 8.5 x 10⁸/mm³)], leucopenia [leukocytes: 4,700/mm³ (reference values: 5,000 - 10,000/mm³)], and thrombocytopenia [platelet: 19,000/mm³ (reference values: 200,000 - 500,000/mm³)]. Blood was collected in EDTA for PCR, and in heparin for cell culture isolation.

### Isolation and cultivation of Ehrlichia spp.

Heparin-anticoagulated whole-blood was collected aseptically from the jugular vein and processed according to Paddock et al. (20). Briefly, leukocytes were isolated by overlaying the buffy coat on Histopaque 1083 (Sigma Diagnostic, St Louis, Mo.), and the interface containing the leukocyte fraction was collected and resuspended in 5 ml of Dulbecco’s Modified Eagle’s medium (Sigma Aldrich, St Louis, Mo.) supplemented with 10% heat-inactivated bovine calf serum (Hyclone Laboratories, Logan, Utah). The leukocyte suspension was transferred to a 25 cm² flask culture and incubated at 37°C in a 5% CO₂ atmosphere. After 24 hrs, 2 ml of fresh culture medium was added and the cells harvested 24 hrs later and added to a monolayer of uninfected DH82 cells (provided by Jere W. McBride, University of Texas Medical Branch) in a 25 cm² flask. The culture was maintained under the same conditions as above, except that the bovine calf serum was reduced to 2.5%, and the culture medium was partially (20%) replaced every 2-3 days. Cell cultures were monitored twice a week for presence of morulae by both Diff-Quik staining (Laborclin, Pinhais, PR, Brazil) and presence of ehrlichial DNA using PCR.

### DNA extraction from blood and cultured cells

DNA was extracted from either 200 μl of canine blood or from inoculated DH82 cells by the DNA easy Tissue Kit (Qiagen Incorporation, Valencia, Calif) according to the manufacturer’s protocol. Each DNA sample was eluted in 100 μl of TE buffer and stored at -20°C until use for PCR.

### PCR analyses

Extracted DNA (5 μl containing 100-200 ng of DNA) from infected cell cultures was used as a template to amplify fragments of ehrlichial dsb, 16S rRNA, and p28 genes. DNA obtained from infected dog blood was used to amplify a fragment of the ehrlichial dsb. For the dsb gene, a 409-bp fragment was amplified with primers DSB-330 (5’-GAT GAT GTC TGA AGA TAT GAA ACA AAT-3’) and DSB- 728 (5’-CTG CTC GTC TAT TTT ACT TCT TAA AGT-3’), as previously described (10) with some modifications (1). Primers GE2’F2’ (5’- GTT AGT GGC AGA CGG GTG AGT-3’) and HE3 (5’-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3’) were used to amplify a 360-bp fragment of the 16S rRNA gene (4, 5). Primers 793´(5’-GCA GGA GCT GTT GTT TAC TC-3’) and 1330 (5’-CCT TCC TCC AAG TTC TAT GCC-3’) were used to amplify a 518-bp portion of the p28 gene (17). PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and examined by UV transillumination. To minimize the potential risks for contamination, DNA extraction, PCR, and agarose gel electrophoresis were performed in physically separate rooms. Positive (DNA extracted from tissue cultured-E. chaffeensis) and negative controls (water) were included in all PCR assays.

### Sequence analyses

PCR amplicons were purified using ExoSAP-IT® (USB, Cleveland, USA) and sequenced directly with both forward and reverse primers of each gene (dsb, 16S rRNA and p28) with an automated ABI Prism 310 Genetic Analyser (Applied Biosysts/Perkin Elmer). The BLAST program (National Center for Biotechnology Information, Bethesda, MD) was used to compare dsb, 16S rRNA and p28 sequences in order to determine sequence similarities. Deduced amino acid sequences of DSB and 28-kDa proteins were analyzed by using the BLAST program.

### RESULTS

#### Isolation and culture from dog blood

PCR amplification of the ehrlichial dsb gene fragment yielded expected amplicon size in DNA extracted from the dog blood and from the DH82 monolayer 14 days after inoculation with the primary leukocyte culture. On day 21, ehrlichial morulae were observed within the cytoplasm of cells (1-5% of infected cells). On day 28, 5% of the cells were infected by visualization of ehrlichial morulae (Fig. 1) and E. canis DNA was detected in the culture. The isolate replicated relatively slowly, reaching 90-100% of infected DH82 cells only after three months. Subsequently, infected cells were sub cultured to uninfected DH82 cells, and the infection reached 90-100% in 10 to 14 days. Thereafter, the infected cells were sub cultured several times, reaching 90-100% infection rates in 7 to 10 days after inoculation. Frozen stocks of infected DH82 cells (left at -80°C for several months) maintained a high infectivity when thawed and added to uninfected DH82 monolayers.

The E. canis isolate, designated São Paulo strain, has been deposited in the Rickettsial Collection of the Laboratory of Parasitic Diseases of the Faculty of Veterinary Medicine, in the University of São Paulo (under M.B.L.), and in the reference collection of the UTMB Rickettsial and Ehrlichial Diseases Research Laboratories (maintained by Jere W. McBride) where they are available upon request.
Molecular characterization of the ehrlichial isolate

DNA extracted from infected DH82 cells was used as template for PCR targeting portions of the ehrlichial genes dsb, 16S rRNA, and p28. Expected amplicons were visualized in all reactions. After DNA sequencing, generated sequences (excluding corresponding primer regions) contained 355, 339, and 484-bp for dsb, 16S rRNA, and p28 genes, respectively. Sequences showing highest identity to the Sao Paulo isolate generated sequences were determined by BLAST analyses (Table 1). The isolate exhibited high identity with corresponding sequences of *E. canis* in GenBank, confirming that the isolate was *E. canis*. Deduced amino acid sequence from partial dsb sequence was 100% (118/118) identical to *E. canis* strain Jake, 83.8% (99/118) to *E. chaffeensis*, 79.6% (94/118) to *E. muris*, 78.8% (93/118) to *Ehrlichia* sp strain IOE, 74.5% (88/118) to *E. ewingii*, and 72.8% (86/118) to *E. ruminantium*. Deduced amino acid sequence from partial p28 sequence was 99.3% (160/161) identical to *E. canis* strain Jake, 98.7% (159/161) to *E. canis* strains Oklahoma and VHE, 81.9% (132/161) to *E. chaffeensis*, 72.6% (117/161) to *E. muris*, 63.7% (65/102) to *E. ewingii*, and 58.3% (91/156) to *E. ruminantium*. Partial sequences (dsb, 16S rRNA and p28) from *E. canis* strain São Paulo generated in this study were deposited into GenBank and assigned the nucleotide accession numbers DQ460713, DQ460714, and DQ460715.

Figure 1. *Ehrlichia* morulae (São Paulo strain) within DH82 cells stained by Diff-Quik staining (original magnification 100x).

### Table 1. Nucleotide sequence identities of the dsb, 16S rRNA and p28 partial sequences of *E. canis* São Paulo strain with available *E. canis* sequences.

| *Ehrlichia* species/ isolate | Gene sequence identity % (number of identical nucleotides / total nucleotides) | dsb | 16S rRNA | p28 |
|----------------------------|--------------------------------------------------------------------------------|-----|---------|-----|
| *E. canis* Jaboticabal       | 100 (355/355) [DQ460716]                                                        | Na  | Na      | Na  |
| *E. canis* VDE               | Na                                                                              | 100 (339/339) [AF373613] | Na   |
| *E. canis* VHE               | Na                                                                              | 100 (339/339) [AF373612] | 99.5 (482/484) [AF165815] |
| *E. canis* Florida            | Na                                                                              | 99.7 (338/339) [M73226] | Na   |
| *E. canis* Jake              | 100 (355/355) [AF403710]                                                        | 100 (339/339) [CP000107] | 99.8 (483/484) [CP000107] |
| *E. canis* Oklahoma          | Na                                                                              | 99.7 (338/339) [M73221] | 99.5 (482/484) [AF078553] |
| *E. canis* Israel            | Na                                                                              | 99.7 (338/339) [U26740] | Na   |
| *E. canis* Madrid            | Na                                                                              | 100 (339/339) [AY394465] | Na   |
| *E. canis* Kagoshima         | Na                                                                              | 100 (339/339) [AF536827] | Na   |
| *E. canis* Camarões          | 100 (355/355) [DQ124254]                                                        | Na  | Na      | Na  |
| *E. canis* Germishuys        | Na                                                                              | 99.7 (338/339) [U54805] | Na   |
| *E. chaffeensis* Arkansas    | 81.8 (288/352) [CP000236]                                                       | 98.5 (334/339) [AF416764] | 78.5 (351/447) [AF479833] |
| *E. chaffeensis* Jax         | 81.8 (288/352) [AF403711]                                                       | 98.5 (334/339) [ECU86664] | 81.8 (384/469) [AF479840] |
| *E. ewingii*                 | 76.5 (268/350) [AY428950]                                                       | 98.2 (333/339) [AY093439] | 79.7 (126/158) [AF287961] |
| *E. muris*                   | 79.6 (282/354) [AY236484]                                                       | 97.0 (329/339) [U15527]  | 82.4 (281/341) [AB178804] |
| *E. ruminantium*             | 74.0 (259/350) [AF308669]                                                       | 97.0 (329/339) [CR925678] | 75.6 (189/250) [AF368012] |
| *Ehrlichia* IOE              | 79.3 (281/354) [AY236485]                                                       | 97.3 (330/339) [AB028319] | 82.9 (283/341) [AB178802] |

Na: not available; [ ] GenBank accession number.
DISCUSSION

CME is a very important infectious disease of dogs in Brazil. Anecdotal observations suggest that this agent is widely distributed all over the country, especially in the urban areas, where the tick vector *R. sanguineus* is highly prevalent (14). Isolation and molecular characterization of *E. canis* strain São Paulo demonstrated a high degree of conservation with other *E. canis* strains that are globally distributed. Partial gene sequences of the *E. canis* São Paulo showed it to be nearly identical to other characterized *E. canis* strains. In fact, partial dsb and 16S rRNA sequences were identical to three and five other *E. canis* strains, respectively, from different countries and continents, including North America, Africa, Asia and Europe. However, the p28 partial sequence for *E. canis* São Paulo differed by 1, 2, and 2 nucleotides from the corresponding sequences of the *E. canis* strains Jake (from USA), Oklahoma (USA), and VHE (Venezuela), respectively. This result is expected since both dsb and 16S rRNA genes are highly conserved genes, while p28, which encodes for an immunodominat protein, may be under immune pressure of different hosts and thus exhibit some variation (10,11,18,23).

Our *E. canis* isolate derived from a dog that had been treated with doxycycline and imidocarb for three weeks prior to the recurrence of the clinical CME symptoms. Ineffectiveness of doxycycline for complete elimination of the *E. canis* infection has been reported in dogs (12). On the other hand, the dog’s owner reported tick infestation on the dog during and after the course of antibiotic therapy, what might have favored the recurrence of the disease. Laboratory findings demonstrated low hematological values for all cellular series, resulting in pancytopenia. Notably, platelet count value (19,000/mm³) was much lower than the minimal reference value (200,000/mm³) indicating a severe thrombocytopenia. Based on this clinical and epidemiological history, it is not possible to determine whether recurrence of clinical CME was due to a new infection acquired through infected ticks or the dog harbored chronic form of CME.

The results in this study point out that, currently, *E. canis* is the only recognized *Ehrlichia* species infecting dogs in Brazil. Further studies are required to evaluate other *E. canis* isolates from different geographic areas of Brazil, and verify the presence of different *Ehrlichia* species infecting dogs. For instance, *E. chaffeensis* DNA was recently reported in wild marsh deer (*Blastocerus dichotomus*) from the eastern part of the state of São Paulo (16).

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