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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 an 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 history

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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 imidorcarb 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 10^6/mm^3 (reference values: 5.5 - 8.5 x 10^6/mm^3)], leucopenia [leukocytes: 4,700/mm^3 (reference values: 5,000 - 10,000/mm^3)], and thrombocytopenia [platelet: 19,000/mm^3 (reference values: 200,000 - 500,000/mm^3)]. 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 cm^2 flask culture and incubated at 37ºC 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 cm^2 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 -20ºC 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 GGT 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 eletrophoresis 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 Biosystens/Perkin Elmer). The BLAST program (National Center for Biotechnology Information, Bethasda, 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 to14 days. Thereafter, the infected cells were sub cultured several times, reaching 90-100% infection rates in 7 to10 days after inoculation. Frozen stocks of infected DH82 cells (left at -80ºC 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.

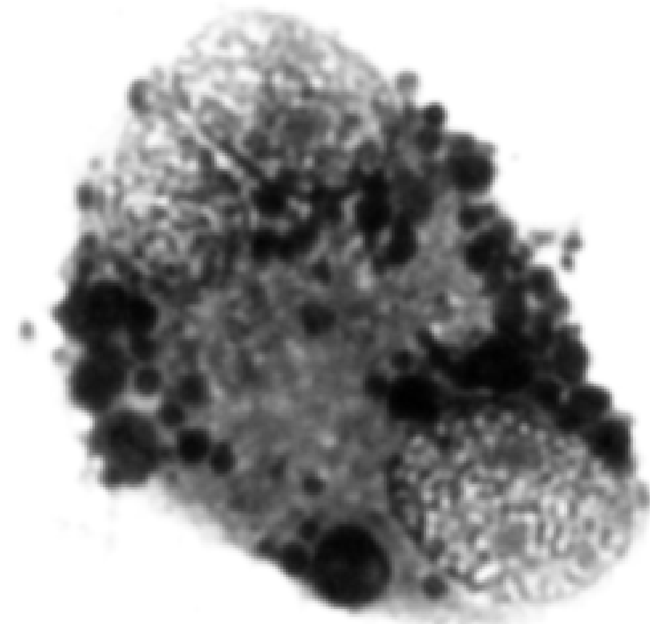

Figure 1. *Ehrlichia* morulae (São Paulo strain) within DH82 cells stained by Diff-Quik staining (original magnification 100x).

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
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
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 imidorcarb 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/mm^3) was much lower than the minimal reference value (200,000/mm^3) 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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RESUMO

Isolamento e caracterização molecular de um isolado de *Ehrlichia canis*

Foi obtido um isolado de *Ehrlichia canis* a partir de um cão naturalmente infectado com sinais clínicos de erliquiose, oriundo do município de São Paulo, SP, Brasil. O isolado foi caracterizado molecularmente pela PCR e seqüenciamento de porções dos genes *dsb*,16S rRNA, e *p*28. A seqüência parcial dos genes *dsb* e 16Sr RNA apresentaram-se idênticas a três e cinco seqüências respectivamente, de *E. canis* provenientes de diferentes países e continentes (América do Norte, África, Ásia e Europa). Contrariamente, a seqüência parcial do gene *p*28 do isolado São Paulo diferiu em um nucleotídeo do isolado Jake (EUA) e dois nucleotídeos dos isolados Oklahoma (EUA) e VHE (Venezuelan Human Ehrlichia – Venezuela). Atualmente, a *E. canis* é a única espécie de *Ehrlichia* que acomete cães no Brasil.

Palavras-chaves: *Ehrlichia canis*, Cão, Caracterização molecular, Erliquiose.

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