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Genetic diversity of *Ehrlichia canis* in Brazil

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ABSTRACT

Canine monocytic ehrlichiosis is a highly prevalent disease in Brazil, where the genetic diversity of *Ehrlichia canis* remains undefined. In this study, we used the TRP36 gene to examine the genetic diversity of *E. canis* strains from naturally infected dogs residing in five distinct geographic regions in Brazil. *E. canis* DNA was detected in 82/126 (65%) dogs by *dsb*-specific PCR and *E. canis* was isolated in cell culture from 13 dogs. Sequences obtained from *dsb* genes amplified from the isolates were identical to the US *E. canis* strain. An extended molecular characterization based on the *TRP36* gene identified two major genogroups based on differences among eight isolates. Isolates with tandem repeat amino acid sequence (TEDSVSAPA) identical to the previously reported TRP36 sequence were found in the midwest, northeast and southeast regions of Brazil, and classified into the US genogroup. A novel Brazilian genotype with a different tandem repeat sequence (ASVVPEAE) was also identified in midwest, northern and southern regions. Similarity in the N-terminal sequence of a US genogroup member with the Brazilian genogroup suggested that genomic recombination between the two genogroups may have occurred. Other subtypes within the Brazilian genogroup were also identified using C-terminal amino acid divergence. We identified two distinct major Brazilian genogroups and several subtypes based on analysis of *TRP36*, and such information will be useful for further genotyping and possible associations with disease severity, understanding of the genetic and antigenic variability of *E. canis*, and for developing strain-specific vaccines and diagnostic methods based on TRP36.

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1. Introduction

Canine monocytic ehrlichiosis (CME) is a globally distributed, potentially fatal tick-borne rickettsial disease of dogs caused by *Ehrlichia canis* (Dumler et al., 2001). CME is endemic in Brazil, highly prevalent among dogs throughout the country (Vieira et al., 2011). Despite the serologic evidence of widespread *E. canis* infections in Brazil, little is known regarding the molecular characteristics of Brazilian *E. canis*. Molecular characterization of several genes from the São Paulo strain of *E. canis* has suggested that *E. canis* strains in North and South Americas are conserved (Aguiar et al., 2008).

Despite the worldwide distribution of *E. canis*, 16S rRNA gene sequences are 99.4–100% identical among isolates from South America, North America, Asia, Europe, Africa, and Middle East. The close similarity between *E. canis* 16S rRNA genes provides little information regarding the overall diversity of this organism and suggests a high level of conservation. Similarly, the immunoreactive proteins including the OMP-1 family, Dsb, TRP19, and TRP140 have also been found to be conserved in geographically dispersed strains (Aguirre et al., 2004; Yu et al., 2007; Aguiar et al., 2008; Zhang et al., 2008; Huang et al., 2010; Kamani et al., 2013). However, differences in the *TRP36* gene have been reported, indicating some degree of *E. canis* diversity in nature, and studies have suggested that *TRP36* is useful for genotyping *E. canis* strains based on differences in tandem repeat number or sequences (Doyle et al., 2005a; Hsieh et al., 2010; Kamani et al., 2013).

E. canis TRP36 contains a major antibody epitope in the tandem repeat region (Doyle et al., 2006) and ehrlichial TRPs are major immunoreactive proteins that have been associated with functional host–pathogen interactions such as adhesion and internalization, actin nucleation, and immune evasion (McBride and Walker, 2011). Examination of the TRP36 gene in US, Brazilian (only a single strain), Cameroonian, Nigerian, and Taiwan *E. canis* strains identified variations in the number of tandem repeats, even though the nine-amino acid repeat sequence (TEDSVSAPA) was shown to be conserved. Notably, a divergent *E. canis* genotype has been identified in Israel based on differences in the TRP36 tandem repeat which was resulted in differences in the immunoreactivity of TRP36 (Zhang et al., 2008).

As CME is a prevalent disease in Brazilian dogs, efforts to understand the molecular and antigenic diversity of the organism will undoubtedly contribute to understanding microbial factors associated with disease pathogenesis as well as developing reliable immunodiagnostics and effective vaccines. In this investigation, we obtained numerous new *E. canis* isolates from dogs representing various regions of Brazil, and examined their genetic diversity based on amino acid sequences of TRP36.

2. Materials and methods

2.1. Blood collection and Ehrlichia isolation

Blood samples were collected from 126 dogs presenting at Veterinary Hospitals and Zoonosis Control Centers from various regions in Brazil (north, midwest, northeast, southeast, and south regions) from January 2007 to February 2012. The dogs exhibited clinical signs consistent with CME. Blood samples were collected with EDTA or heparin as an anticoagulant. *E. canis* PCR positive samples (45/82) that did not demonstrate hemolysis were inoculated into cell culture (DH82 cells) to isolate the organism as previously described (Aguiar et al., 2008).

2.2. PCR amplification of major immunoreactive protein gene

Genomic DNA from whole blood was extracted using a commercial kit according to the manufacturer's protocol (MO BIO Laboratories Inc., Carlsbad, CA). For molecular diagnosis, PCR amplification of a partial *dsb* gene sequence was performed according to Doyle et al. (2005b). For cell culture isolates, genomic DNA was purified and amplified by PCR with 5 PRIME HotMasterMix (5 PRIME, Gaithersburg, MD) and universal primers TRP36-F2 (5′-TTTAAAACAAAATTAACACACTA-3′) and TRP36-R1 (5′-AAGATTAACTTAATACTCAATATTACT-3′) in order to obtain full *TRP36* gene sequences. DNA templates were amplified using a thermocycling protocol of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min for 30 cycles. Single PCR amplicons (ranging from 800 to 1000 bp) were visualized by agarose gel electrophoresis (1.2% FlashGel, Lonza).

2.3. DNA sequencing and phylogenetic analysis

TRP36 amplicons were purified using a PCR purification kit (Qiaquick, Qiagen) and sequenced directly using the same PCR primers at the University of Texas Medical Branch Protein Chemistry Laboratory. The *TRP36* gene from each isolate was amplified and sequenced multiple times from independent reactions to confirm the sequence fidelity. The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used for the comparison and the analysis of sequence data obtained in this study with 14 *E. canis* and two *E. chaffeensis* isolates previously deposited in GenBank database (*E. canis*: Brazil – São Paulo – ABA39257; Israel – Ranana – ABW91006; Israel – ABV02078; Africa – Cameroon – ABA39258, United States (US) – Jake – AAZ40199, United States (US) – Oklahoma – AAZ40200, Taiwan TWN1 – ABS82573, Taiwan TWN2 – ABU44524, Taiwan TWN3 –ABV26011, Africa – Nigeria 64 – JN622143, Africa – Nigeria 80 – JN982338, Africa – Nigeria 94 – JN982341; *E. chaffeensis*: United States (US) – Arkansas – AAZ40202, and Sapulpa – DQ085431). Nucleic acid and amino acid alignments, percent identity, and phylogenetic relationships were determined with the MEGA Beta program (Tamura et al., 2011).

3. Results

3.1. Isolation and culture of E. canis from dog blood

DNA of the *E. canis dsb* gene was amplified from 82 (65%) dogs in this study. *E. canis* was isolated from 13/45 dogs within 30 days of inoculation into cell culture as determined by microscopic detection of intracellular inclusions and confirmation by *dsb* PCR. Partial sequences