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First isolation and molecular characterization of *Ehrlichia canis* in Costa Rica, Central America

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ABSTRACT

The present study investigated *Ehrlichia* species in blood samples from dogs suspected of clinical ehrlichiosis, using molecular and isolation techniques in cell culture. From a total of 310 canine blood samples analyzed by 16S rRNA nested PCR, 148 (47.7%) were positive for *Ehrlichia canis*. DNA from *Ehrlichia chaffeensis* or *Ehrlichia ewingii* was not detected in any sample using species-specific primers in separated reactions. Leukocytes from five PCR-positive dogs were inoculated into DH82 cells; successful isolation of *E. canis* was obtained in four samples. Partial sequence of the *dsb* gene of eight canine blood samples (including the five samples for *in vitro* isolation) was obtained by PCR and their analyses through BLAST showed 100% of identity with the corresponding sequence of *E. canis* in GenBank. This study represents the first molecular diagnosis, isolation, and molecular characterization of *E. canis* in dogs from Costa Rica.

Ehrlichiosis is a tick-borne disease caused by an obligatory intracellular bacterium with special tropism for endothelial, neutrophile, monocyte and macrophage cells (Dumler et al., 2001). *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Ehrlichia ewingii* are reported to cause disease in dogs and humans (Anderson et al., 1992; Breitschwerdt et al., 1998; Buller et al., 1999; Pérez et al., 2006). *E. canis* is distributed worldwide, and has been detected by molecular techniques in Israel, United States, Brazil, Spain, Mexico and Portugal (Keysary et al., 1996; Murphy et al., 1998; Dagnone et al., 2003; Aguirre et al., 2004; Hori-Oshima et al., 2006; Alexandre et al., 2009). *E. chaffeensis* and *E. ewingii* are identified as cause of human and dog disease in United States (Anderson et al., 1992; Breitschwerdt et al., 1998; Buller et al., 1999); *E. ewingii* DNA was also determined in dogs from Cameroon (Ndip et al., 2005). Based on clinical, microscopic evaluation and immunofluorescence assay canine ehrlichiosis was reported for the first time in 1995 in Costa Rica, since then, a wide distribution of *E. canis* infection in dogs has been reported based on hematological and serological results (Meneses, 1995; Rímolo, 2006). However, microscopic evaluation of stained blood smears are not specific and sensitive enough, while serological results did not discriminate between past and current infections (Breitschwerdt et al., 1998; Buller et al., 1999). Finally, both techniques did not determine the species infecting the host. The objective of the present study was to determine *Ehrlichia* species infecting dogs from Costa Rica by molecular techniques and isolation in cell culture.

Assuming to detect at least 1% of infected dogs, a total of 310 blood samples from dogs with suspected clinical ehrlichiosis were collected from three veterinary laboratories from October 2006 to October 2007 (total number of dogs in the Central Valley of Costa Rica >40,000, confidence level of 95% confidence, 1% expected prevalence, Cannon and Roe, 1982). The samples were collected into plastic tubes containing EDTA and stored at −20 °C until DNA extraction and molecular analysis were carried out.

DNA extraction of whole blood was carried out using Wizard Genomic Purification Kit (Promega Corporation, Madison, WI, USA) and nested PCR was carried out as described previously expecting amplicons of about 389-bp (Dawson et al., 1994; Kocan et al., 2000). Plasmids containing segments of DNA from *E. canis*, *E. chaffeensis* and *E. ewingii* were used as positive controls, whereas

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water was used as negative control (Sirigireddy and Ganta, 2005). From five dogs that yielded nested PCR-positive results, blood was collected in heparin-sterile tubes and processed for isolation procedures as previously described (Aguiar et al., 2008). In order to amplify a fragment of the ehrlichial *dsb* gene for comparative sequence analysis, PCR was carried out as described by Aguiar et al. (2007) with eight canine samples that resulted positive in the nested PCR, including five samples that were subjected to isolation using cell culture technique. DNA extraction from culture was carried out with DNeasy Blood and Tissue Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instruction. DNA from *E. chaffeensis* was used as positive control and water as negative control. The amplicons about 409 bp were purified for DNA sequencing using EXOSAP-IT (USB Corporation, Cleveland, OH, USA). Both forward and reverse strands of each PCR amplicon were newly amplified with the same primers and then sequenced on an automated sequencer model ABI Prism 310 Genetic Analyser (Applied Biosystems/Perkin Elmer, Foster City, CA, USA). BLAST program (National Center for Biotechnology Information, Bethesda, MD) was used to compare *dsb* sequences in order to determine the species.

E. canis DNA were found in 148 (47.7%) out of 310 samples assayed by the nested PCR. DNA from *E. chaffeensis* or *E. ewingii* was not detected in any sample.

Successful isolation of *E. canis* in cell culture was obtained in four out of five blood samples tested. After 21 days the canine isolates from Costa Rica showed intracytoplasmatic inclusions compatible with *Ehrlichia* in the cytologic assay and yielded positive results by PCR. PCR amplification followed by DNA sequencing of a fragment of the *dsb* gene of eight canine blood samples from Costa Rica (including the five samples processed for *in vitro* isolation) showed sequences identical to each other and to the *dsb* corresponding sequence of *E. canis* in GenBank (AF403710).

The results presented in this study confirm *E. canis* as the causative agent of canine ehrlichiosis in Costa Rica. The high rate (47.7%) of *E. canis*-positive dogs detected in the present study is certainly linked to the sampling method, since most of the analyzed samples were taken from dogs suspected of having ehrlichiosis. Nevertheless, our results are in agreement with previous studies carried out in Latin America, that reported *E. canis* as the only agent found infecting dogs (Labruna et al., 2007; Aguiar et al., 2007).

E. chaffeensis and *E. ewingii* were not detected in the present study and has been reported in dogs in the United States using molecular techniques (Breitschwerdt et al., 1998; Buller et al., 1999). These agents are primarily transmitted by *Amblyomma americanum* ticks, (Anziani et al., 1990; Dawson et al., 1994), whereas *E. canis* is transmitted primarily by *Rhipicephalus sanguineus* ticks. Studies carried out by Abrego (2008) showed that *R. sanguineus* is the main tick of dogs of the Costa Rican Central Valley; thus, 160 (97%) ticks collected from 165 dogs were classified as *R. sanguineus*, four as *Amblyomma ovale* and one as *Ixodes boliviensis*. This could explain why *E. chaffeensis* and *E. ewingii* were not detected in the present study; however, the presence of these agents in other regions from Costa Rica could not be ruled out.

Partial *dsb* sequences of *E. canis* from Costa Rican infected dogs showed to be identical to other *E. canis* corresponding sequences in GenBank, what is in accordance with previous studies that reported a highly conservation of the ehrlichial *dsb* gene among *E. canis* strains from different countries and continents (Doyle et al., 2005; Aguiar et al., 2008). This study reports the first molecular diagnosis and characterization of *E. canis* strains from Costa Rica and confirms this agent as the cause of canine ehrlichiosis in the country. Further researches are required to determine the presence or absence of other species of *Ehrlichia* in Costa Rica.

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