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Original article

Novel genotype of *Ehrlichia canis* detected in samples of human blood bank donors in Costa Rica

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ABSTRACT

This study focuses on the detection and identification of DNA and antibodies to *Ehrlichia* spp. in samples of blood bank donors in Costa Rica using molecular and serological techniques. Presence of *Ehrlichia canis* was determined in 10 (3.6%) out of 280 blood samples using polymerase chain reaction (PCR) targeting the ehrlichial *dsb* conserved gene. Analysis of the ehrlichial *trp36* polymorphic gene in these 10 samples revealed substantial polymorphism among the *E. canis* genotypes, including divergent tandem repeat sequences. Nucleotide sequences of *dsb* and *trp36* amplicons revealed a novel genotype of *E. canis* in blood bank donors from Costa Rica. Indirect immunofluorescence assay (IFA) detected antibodies in 35 (35%) of 100 serum samples evaluated. Thirty samples showed low endpoint titers (64–256) to *E. canis*, whereas five sera yielded high endpoint titers (1024–8192); these five samples were also *E. canis*-PCR positive. These findings represent the first report of the presence of *E. canis* in humans in Central America.

1. Introduction

Human ehrlichiosis are tick-borne zoonosis caused by rickettsial pathogens (order Rickettsiales, family Anaplasmataceae, genus *Ehrlichia*), considered as emerging pathogens in the United States, and increasingly, in many countries around the world (Ismail et al., 2010). *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis, is the most important species to infect humans, and less commonly, *Ehrichia ewingii* (human ewingii ehrlichiosis) (Dumler, 2005). The distribution of these diseases are largely determined by the presence of known competent tick vectors (*Amblyomma americanum*, the lone star tick) and reservoir hosts (*Odocoileus virginianus*, white-tailed deer) (Dumler, 2005). Although these agents may also affect dogs, canine monocytic ehrlichiosis, caused by *Ehrlichia canis,* is best recognized as a canine pathogen, which is transmitted mainly by the brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.) (Dawson et al., 1993; Dumler et al., 2001).

Until recently, *E. canis* was thought to infect only canines; however, it was isolated and molecularly characterized from an asymptomatic human in Venezuela (Perez et al., 1996), although its role as a pathogen was not immediately recognized. In 2006, *E. canis* was detected in human patients showing clinical findings compatible with ehrlichiosis (Pérez et al., 2006). Since then, *E. canis* should be considered as a potential agent of human illnesses in areas endemic for canine monocytic ehrlichiosis, caused by *E. canis* (Diniz et al., 2007; Nicholson et al., 2010).

Once the ehrlichial organisms are introduced into the host, they multiply in monocytes and macrophages (*E. chaffeensis* and *E. canis*) or in peripheral blood neutrophils (*E. ewingii*), and spread to phagocyte cells of different organs such as liver, spleen, lungs and lymph nodes (Dumler et al., 2001). Detection of morulae-like forms of *Ehrlichia* within leukocytes through stained blood smears lead to rapid diagnosis of *Ehrlichia* spp.; however, this detection method is usually efficient only during the initial stages of infection, when there are higher levels of rickettsemia (Chapman et al., 2006). *In vitro* isolation of viable ehrlichial organisms in cell culture remains the gold standard diagnostic method; however, it is time consuming and requires more refined laboratory conditions

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(Dawson et al., 1991; Felek et al., 2001; Gusa et al., 2001). Indeed, the most common diagnostic methods of rickettsial diseases, including ehrlichiosis, rely on serological and molecular assays (Chapman et al., 2006). While the indirect immunofluorescence assay (IFA) is frequently employed in research studies, this serologic method does not allow a precise identification of the *Ehlichia* species that caused infection, since there are serological cross-reactions, for example, between *E. canis, E. chaffeensis* and *E. ewingii*. In addition, serology does not allow determining whether the infection is active or occurred in the past (Breitschwerdt et al., 1998). Molecular tests such as polymerase chain reaction (PCR) lead to rapid and accurate diagnosis, determining the *Ehrlichia* species that could be actively infecting the host (Romero et al., 2010).

Ehrlichia canis was firstly reported infecting dogs in Costa Rica in 1995 (Meneses 1995). Since then, several studies have provided molecular detection of *E. canis* in blood samples of domestic and wild animals and their ticks in the country (Barrantes-González et al., 2016; Campos-Calderón et al., 2016; Dolz et al., 2015; Rojas et al., 2014; Romero et al., 2011). In the past, four clinical cases of ehrlichiosis were reported in humans in Costa Rica, based on clinical evaluation and microscopic detection of intracytoplasmic inclusions in granulocytes (Hernández de Mezerville and Padilla-Cuadra, 2007; Rojas-Solano and Villalobos-Vindas, 2007) and monocytes (Brenes et al., 2011) in peripheral blood. Recently, Rojas et al. (2015) reported human natural infection by *E. chaffeensis* in patients in the Northern region of Costa Rica. In the present work, we present evidence of *Ehrlichia* infection in blood bank donors, including the first report of a novel genotype of *E. canis* associated with humans in Costa Rica.

2. Material and methods

2.1. Population studied

Blood samples from blood bank donors collected during 2007 were analyzed by PCR targeting ehrlichial DNA. In addition, 100 serum samples, randomly selected from the same bank donor population, were tested by IFA for the presence of *E. canis*-reactive antibodies. The minimum sample size for detecting infection with an expected prevalence of 1% (95% confidence) in a population of 10,000 donors was 294. For logistics reasons the number of samples analyzed by PCR was 280, which allows detecting a 1.1% infection prevalence.

According to universal criteria defined by Costa Rican blood banks, blood donors were 18–60 years old, weighed more than 50 kg, and presented hemoglobin >12 g/dl, hematocrit >38%, systolic arterial pressure <160 mmHg and diastolic arterial pressure <100 mmHg. In addition, each blood sample was accompanied by sex and age of the donor.

2.2. Polymerase chain reaction (PCR) and DNA sequencing

Extraction of DNA from blood samples was carried out using the saline extraction method described by Miller et al. (1998). For molecular diagnosis, PCR was carried out to amplify a fragment of disulfide oxidoreductase gene (*dsb*) of *Ehrlichia* spp. (Ndip et al., 2005) using the 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′). Each reaction consisted of 12.5 μl of Dream Taq™ PCR Master Mix 2X (Fermentas®), 1.0 μl of each primer (10 pmol/μl), 5 μl of DNA (20 ng) and 5.5 μl of water (molecular biology grade, Fermentas®) in a final volume of 25 μl. Amplification was carried out by denaturalization at 95 °C for 2 min, 40 cycles of denaturalization (30 s, 95 °C), annealing (30 s, 58 °C), extension (30 s, 72 °C), and a final extension at 72 °C for 5 min. Genomic DNA of *E. canis* (KU534872) from a *Rhipicephalus sanguineus* s.l. tick from Costa Rica (Campos-Calderón et al., 2016), and *E. chaffeensis* and *E. ewingii* plasmids (Sirigireddy and Ganta, 2005) were used as positive controls; nuclease free water (Fermentas®) was used as a negative control. The products obtained in PCR were visualized by 2% agarose gel Tris-Borate-EDTA electrophoresis, stained with DNA GelRed (Biotium®). Thermo Scientific GeneRuler 100 bp Plus DNA Ladder (Sm0321) molecular weight marker was included. Samples yielding visible PCR products compatible with 409 bp were considered positive for *Ehrlichia* sp.

Samples positive by the *dsb*-PCR assay were tested by a heminested PCR targeting a segment of the Tandem Repeat Protein 36 gene (*trp36*). In the first reaction, primers TRP36-F2 (5′-TTTAAAACAAAATTAACACACTA-3′) and TRP36-R1 (5′-AAGATTAACTTAATACTCAATATTACT-3) were used (Aguiar et al., 2013). Each reaction consisted of 12.5 μl of Dream Taq™ PCR Master Mix 2X (Fermentas®), 3.0 μl of each primer (10 pmol/μl), 4 μl of DNA (20 ng) and 2.5 μl of water (molecular biology grade, Fermentas®) in a final volume of 25 μl. Amplification was carried out by denaturation at 95 °C for 5 min, 35 cycles of denaturation (30 s, 95 °C), annealing (30 s, 52 °C), extension (1 min, 72 °C), and a final extension of 72 °C for 5 min. In the nested reaction TRP36-R1 and TRP36-D_F (5′-CACACTAAAATGTATAATAAAGC-3′) were used (Aguiar et al., 2014), with the same conditions as described above, but changing annealing temperature (57 °C). PCR products were visualized in 2% agarose gel Tris-Borate-EDTA electrophoresis, stained with DNA GelRed (Biotium®). Thermo Scientific GeneRuler 100 bp Plus DNA Ladder (Sm0321) molecular weight marker was included. Samples yielding PCR products corresponding to 1000 bp (first reaction) and 600 bp (nested reaction) were considered positive for *Ehrlichia* sp.

PCR products were purified using the QIAquick® kit (QIAGEN), and sent to Macrogen (Seoul, Korea) for DNA sequencing. Partial sequences were aligned with BioEdit Sequence Aligment Editor® (Hall, 1999) and compared using the BLASTn algorithm with the database of NCBI (National Center for Biotechnology Information).

2.3. Indirect immunofluorescence assay (IFA)

Anti-*Ehrlichia* spp. antibodies were evaluated by IFA according to Aguiar et al. (2007), using *Ehrlichia canis* São Paulo isolate as antigen. Anti-human IgG secondary antibody (Caltag Laboratories) was used at the 1:200 dilution. A positive control serum was obtained from an experimentally infected dog, as well as a negative control serum from an uninfected dog (Aguiar et al., 2007). Reactive sera at the serum dilution 1:64 were considered seropositive, and subjected again to IFA testing with 2-fold serial dilutions in PBS, from 1:64 to 1:8192, to determine the final endpoint titer.

2.4. Statistical analysis

The frequencies of positive results by PCR and IFA tests were compared by using the Fisher's exact or chi-square test. They were also analyzed in 2 × 2 tables in the Egret epidemiological program version 2.0.3 (Cytel Software Corp.) to evaluate the epidemiological association of the presence of *E. canis* DNA or *E. canis*-reactive antibodies with donor sex or age. For PCR results, the variable "age" was classified by mean (younger or older than 29 years), median (younger or older than 27 years), and three specific arbitrary age strata (<25, between 26 and 35, and >36 years old).

3. Results

Among 280 blood analyzed samples, 10 (3.6%) yielded amplicons of the expected size by the *dsb* PCR. DNA sequences generated from these amplicons exhibited high identity with corresponding *E.*

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canis (AF403710). Sequences of samples 5, 7, 9, 13, 15 were identical to each other (base sequence CR1) and 100% (400/400 bp) identical to *E. canis* (AF403710), while samples 1, 2, 3, 4 and 10 were identical to each other (base sequence CR2) and presented 99% (397/401 bp) of identity to the *E. canis* sequence (AF403710) available in GenBank. Deduced amino acid sequence from partial *dsb* sequence was 99.2% (131/132 bp) similar to each other and 99.2% and 100% identical to *E. canis* (Genbank AF403710). Sequences of *dsb* gene generated in this study (CR1, CR2) were deposited in GenBank accession number KR732921 and KR732922, respectively.

The *trp36* gene was partially amplified (528–757 bp) and sequenced from four samples (1, 3, 5 and 7). Sequences were identical to each other and the identities obtained were 98.7% (221/224 bp) similar to different strains of *E. canis* from Brazil (JX429924, JX312082, JX312080, JX312079, JX312081), and 95.7% (200/209 bp) with strains from Africa (JN982341, JN982338, JN622143, DQ146155) and Middle East (EU118961, EF636663). According to the amino acid sequence, the N-termini region was 97.8% (89/91) identical to a strain from Brazil classified as the Brazilian genotype (GenBank AFS49963, AFS49962, AFS49965, AFS49964, AFT92033). A new tandem repeat sequence was observed, consisting of four repetitions of 'EASVVPAAEAPQPAQQTEDEFFSDGIEA' followed by a fifth sequence with the amino acids 'EASVVPAAEAPQPAQQTEDEFFSDGIE'. Interestingly, the first six amino acids are similar to the Brazilian genotype 'ASVVPEAE'. This last repetition sequence presented a deletion of Alanine in the last position before a new C-termini region sequence with amino acid sequence of 'EVLSAFL'. A phylogenetic tree (Fig. 1) of *E. canis* TRP36 was performed with sequences of this study, and sequences available in GenBank. The Costa Rican amino acid sequence grouped in a clade with the Brazilian genogroup, previously detected in Brazilian dogs. Sequence of TRP36 gene generated in this study was deposited in GenBank under accession number KU194227.

Statistical association was not found when age was classified by median (27 years). However, when age was classified by the mean (29 years), donors >29 years old had 5.6 times more risk to have *E. canis*-PCR positive results than donors ≤29 years old (p = 0.006; IC 95%: 1.3–28.3). In the arbitrary age stratification, there were no strata with statistically significant risks; however, an infection tendency risk was observed as age increased (Table 1). Overall, 45.3% (127/280) blood samples were from males, from which six were PCR-positive. Females were represented by 153 samples, with four PCR-positive. Statistical analysis did not show association between PCR results and age or sex (p = 0.52).

Among 100 serum samples analyzed by IFA, 35 were reactive to *E. canis* antigens. Thirty of them presented relatively low endpoint titers (64–256), while five showed high endpoint titers (1024–8192); these five samples also showed PCR positive results. Among seropositive samples, 11 were from male donors, all <36 years old; 24 were female donors, mostly (23/24) <36 years old.

When the results of PCR and IFA obtained from the 100 human donors were compared, 64 showed negative results in both techniques, while 30 were IFA positive (titers ranging from 64 to 256) and PCR negative; five donors were IFA (titers from 1024 to 8192) and PCR positive. Only one donor was PCR positive and IFA nega-

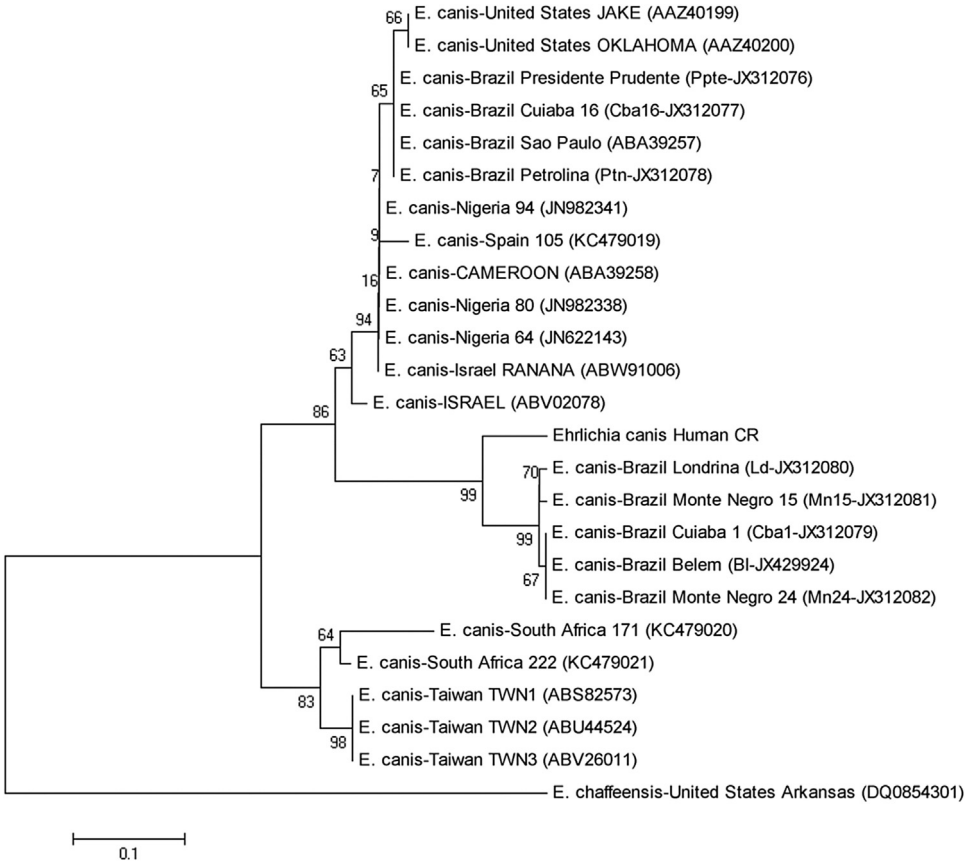

Fig. 1. Phylogenetic relationships between the novel *Ehrlichia* genotype (named as '*Ehrlichia canis* Human CR') and *E. canis* TRP36 amino acid sequences from geographically dispersed *E. canis* strains. Amino acid alignments, percent identity, and phylogenetic relationships were determined with the MEGA Beta program (Tamura et al., 2011). The phylogenetic tree was inferred by the neighbor-joining method with distances calculated by means of the Kimura two-parameter method as the number of base substitution per site. The orthologous TRP47 sequence of *E. chaffeensis* (Doyle et al., 2005) was used as outgroup. All positions containing gaps and missing data were eliminated. The numbers at the nodes represent the percentage of 1000 bootstrap resamplings.

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Table 1
Distribution of PCR results by age, stratified by median, mean and arbitrary stratification defined by the researchers.

Age[1] (years)	Positive	Total	OR	IC (95%)
≤27	3 (2.0%)[a]	150		
>27	7 (5.4%)[a]	130	2.8	0.63–13.90
Age[2]	Positive	Total		
≤29	3 (1.6%)[a]	194		
>29	7 (8.1%)[b]	86	5.6	1.30–28.30
Age[3]	Positive	Total		
≤25	2 (1.9%)[a]	101		
26–35	5 (3.5%)[a]	144	1.78	0.34–9.36
≥36	3 (8.6%)[a]	35	4.64	0.74–29.02

Proportions bearing different superscript letters differ significantly ($p < 0.05$).
[1] Age classified in two strata according to the median.
[2] Age classified in two strata according to the arithmetic median.
[3] Age classified in three strata according to arbitrary criteria of the researchers.

tive; the remaining four PCR positive samples were not analyzed by IFA because they were not selected in the sub-sample due to budget reasons.

4. Discussion

Molecular detection and identification of *E. canis* in blood bank donors of Costa Rica is reported for the first time. Similarly to previous studies with canine or tick samples from Costa Rica (Barrantes-González et al., 2016; Campos-Calderón et al., 2016; Dolz et al., 2015; Rojas et al., 2014; Romero et al., 2011), we did not detect *E. chaffeensis* or *E. ewingii* DNA. While there is a single report of molecular diagnosis of *E. chaffeensis* in human patients in the Northern zone of Costa Rica (Rojas et al., 2015), our results indicate *E. canis* as the most prevalent *Ehrlichia* species infecting humans in the Country, similarly to the reports on animals (Barrantes-González et al., 2016; Campos-Calderón et al., 2016; Dolz et al., 2015; Meneses, 1995; Rojas et al., 2014; Romero et al., 2011). Human infection by *E. canis* in the present study was confirmed by molecular analysis of genes encoding two different proteins; *dsb* a conservative protein that has been used to distinguish different species of *Ehrlichia,* and TRP*36,* a major immunoreactive protein useful for genotyping *E. canis* strains based on differences in tandem repeat number or sequences (Aguiar et al., 2013). Our analysis of later gene indicate that human ehrlichiosis in Costa Rica could be associated to a novel genotype of *E. canis* based on the amino acid tandem repeat sequence of the TRP36 protein. The N-termini sequence of *E. canis* from our TRP36 protein showed 98% similarity to different Brazilian strains and this level of similarity is higher than observed against strains from Brazil and USA (Aguiar et al., 2013). Moreover, a novel amino acid sequence was observed in the repetition region of the protein. Phylogenetic tree inferred with TRP36 sequences generated in the present study showed that *E. canis* genotype detected in humans in Costa Rica grouped within a single clade closely related to the Brazilian genogroup, with high bootstrap support (99%).

Human infection by *E. canis* has been reported in Venezuela and Mexico (Perez et al., 1996; Silva et al., 2014). The presence of *E. canis* in human blood samples in Costa Rica may be a result of the high prevalence of the agent in canines and their ticks (Barrantes-González et al., 2016; Campos-Calderón et al., 2016; Romero et al., 2010, 2011), which increases the likelihood of human exposure to infected ticks. In this context, tick control on dogs is critical to prevent human infection (Barrantes-González et al., 2016). Although *R. sanguineus* s.l. is a typical dog parasite, human parasitism by this tick group has been occasionally reported in many parts of the world, including Latin America (Goddard, 1989; Serra-Freire et al., 2011; Dantas-Torres et al., 2005, 2006; Guglielmone et al., 2006).

Interestingly, results of our serological analysis suggest that a high level (30%) of blood donors seemed to have had contact in the past with *E. canis,* although cross-reactions with *E. chaffeensis* or other *Ehrlichia* species cannot be ruled out (Breitschwerdt et al., 1998). Considering the health status of blood donors in the present study, their likely infection by *E. canis* seemed to occur without disease. This could be due to low virulence of *E. canis* strains circulating in the country, or to the presence of diseases with similar pathologies in the country, which evolve with nonspecific clinical signs, and therefore goes undiagnosed (Perez et al., 1996).

Finally, a tendency towards risk of infection was observed as age increases, which is consistent with Pérez et al. (2006), who reported that higher the age, the greater the probability of having been in contact with an infected tick. The foregoing allows us to raise some important questions: could it be that canine ehrlichiosis, so common in our environment, is responsible for the fact that in Costa Rica human beings are exposed and generate antibodies without this leading to persons contracting the disease? How important are persistent infections in humans? Will they trigger chronic diseases? How important is it that blood with *E. canis* be transfused to diseased or inmunosuppressed persons? Who are the persons most likely to suffer an *E. canis* infection? Further clinical and epidemiological studies are recommened to find answers to all these questions.

5. Conclusions

This study reports the presence of *E. canis,* and specific antibodies against this agent, in the blood of blood bank donors in Costa Rica, which provides the first confirmed diagnosis of *E. canis* in humans in Central America. An increased risk of infection with increased age was found in the studied group of blood donors.

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