Seroprevalence and Genotypic Analysis of *Ehrlichia canis* Infection in Dogs and Humans in Cauca, Colombia

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Abstract. *Ehrlichia canis* infections have been reported in humans in Venezuela and Costa Rica. In this study, 506 healthy residents and 114 dogs from four municipalities (Cauca, Colombia) were surveyed and blood samples collected. Antibodies to *E. canis* in human and canine sera were evaluated using the Tandem repeat protein 19 (TRP19) peptide ELISA and indirect immunofluorescence assay (IFA). *Ehrlichia canis* TRP19 antibodies were detected in only 1/506 human sera, but the single positive sample was negative by IFA. The majority (75/114; 66%) of dogs surveyed had antibodies to the *E. canis* TRP19 peptide by ELISA, and eight randomly selected sera were further confirmed by *E. canis* IFA. Genomic DNA samples obtained from 73 *E. canis* TRP19 ELISA-positive dog blood samples were examined by PCR targeting the 16S ribosomal ribonucleic acid (rRNA) gene. *Ehrlichia canis* 16S rRNA was amplified in 30 (41%) of the dogs, and 16 amplicons were selected for DNA sequencing, which confirmed that all were *E. canis*. A second PCR was performed on the 16 confirmed *E. canis* 16S rRNA PCR-positive samples to determine the TRP36 genotype by amplifying the *trp36* gene. TRP36 PCR amplicon sequencing identified nine dogs infected with the U.S. *E. canis* TRP36 genotype (56%), one dog with the Brazilian genotype (6%), and six dogs with the Costa Rican genotype (38%). Moreover, these molecular genotype signatures were consistent with serologic analysis using TRP36 genotype-specific peptides. Notably, there was no serologic evidence of *E. canis* infection in humans, suggesting that *E. canis* infection in dogs in Cauca is not associated with zoonotic human infection.

INTRODUCTION

Human infections with *Ehrlichia* species are increasingly being diagnosed in several countries, where factors such as the presence and number of reservoir animals and tick vectors in endemic areas could play a critical role in emerging infections.¹ The genus *Ehrlichia* has six recognized species that infect humans or animals: *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia ruminantium*, *Ehrlichia muris*, and *Ehrlichia minasensis*.^{2,3} *Ehrlichia chaffeensis*, *E. ewingii*, *E. canis*, and *E. muris* subsp. *euclairensis* have been identified as causative agents of zoonotic emerging human infections, where the first three agents are also known to cause ehrlichiosis in dogs.^{4,5} *Ehrlichia canis* has been associated with human infections in Venezuela and Costa Rica by serological and molecular tests.^{6–8}

Tandem repeat proteins (TRPs) in *E. chaffeensis* and *E. canis* are major immunoreactive proteins with species-specific linear epitopes in the tandem repeat domain.⁹ In particular, *E. canis* TRP19 and TRP140 are species-specific antigens with exposed, conserved surface domains,^{10–12} whereas the *E. canis* TRP36, is a species-specific antigen with different TR sequences among *E. canis* strains.¹³ Previous studies have demonstrated that TRP19 and TRP36 are excellent immunodiagnostic antigens.^{10,14,15} Tandem repeat protein 36 has been used in many studies to define genotypic differences among *E. canis* strains by sequencing the tandem repeat region which exhibits variability in the nucleic acid sequence and in the number of

tandem repeats.^{6,16–18} Recently, differences in TRP36 epitope containing tandem repeats has been used to perform serogenotypic analysis and has revealed evidence of sequential or coinfections in dogs with multiple *E. canis* strains.^{16,18}

In Colombia, several reports have established and documented the seroprevalence and/or molecular evidence of *E. canis* infection in dogs throughout the country.^{18–22} In addition, there are reports of human ehrlichiosis where cytoplasmic inclusions consistent with ehrlichial morulae have been visualized in peripheral blood smears of humans or *Ehrlichia*-reactive antibodies have been detected by indirect immunofluorescence assay (IFA).^{23,24}

Using dogs as sentinels for human rickettsial diseases has been suggested as an approach for disease surveillance by One Health.²⁵ In this study, we investigated the seroprevalence of *E. canis* infection in humans and dogs in municipalities of Cauca, Colombia, to investigate the prevalence of *E. canis* infection in dogs, characterize *E. canis* genotypes, and investigate the possible occurrence of zoonotic infections in humans who own infected dogs.

MATERIALS AND METHODS

Study population and sample collection. A cross-sectional study was carried out in the Department of Cauca (Colombia, South America). Samples were collected between August and November of 2017 in four municipalities (see Supplemental Table S1). Those municipalities were selected according to previous reports of human tick infestations or tick bites, ecological conditions for development of tick populations, security warrant for the field collection personnel, and ease of access to the rural human populations. To establish an appropriate sample size for the human population,

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census data from each municipality mayor's office was accessed. A confidence level of 95% with a 5% margin of error and a 50% expected prevalence rate for rickettsial infection was used, providing a sample size of 385 people. A predefined epidemiological questionnaire was completed for each healthy adult volunteer to determine risk factors for rickettsial infection. Each dog owner provided information regarding the number of dogs owned, length of ownership, tick infestation, ticks present in the dwelling (presence and infestation duration), and type of treatment used to control ticks, if any.

Healthy human volunteers and their dogs were identified by using a house-to-house campaign. All volunteers signed an informed, explained consent before any information or blood samples were obtained. The research ethics board of the Basic Sciences College, Pontificia Universidad Javeriana, approved the informed consent (Meeting Minute Record No. 9: May 11, 2016). Whole blood and serum samples were collected by venipuncture from the median cubital vein in humans and cephalic vein in dogs. A total of 506 human (a sample size higher than initially calculated of 385 people) and 114 canine sera were collected for this study. All dogs were examined for ectoparasite infestation (ticks, fleas, and lice). The ticks were collected in 70% ethanol and transported to the Veterinary Parasitology Laboratory (Universidad de La Salle) for morphological identification using a stereoscope using standard taxonomic keys.^{26,27}

ELISA. The ELISA to detect *E. canis* TRP19 antibodies has been described previously.^{16,18} In brief, 96-well microtiter plates (MaxiSorp, Nunc®, #50-112-3685, Invitrogen by Thermo Fisher Scientific) were coated overnight with TRP19 peptide (1 µg/well, 50 µL) and diluted in sterile phosphate-buffered saline (PBS). Dog and human sera were diluted (1:100) in blocking buffer (Tris-buffered saline with 0.1%, 20%, and 10% horse serum) and added to the wells (50 µL), and the plates were incubated at room temperature for 1 hour. The plates were washed, and reverse alkaline phosphatase (AP)-labeled secondary antibody conjugated with goat anti-human IgG (H + L) reverse AP-labeled antibody (Kirkegaard and Perry) or anti-dog IgG (H + L) reverse AP-labeled antibody (50 µL; 1:5,000; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD.) were added and incubated at room temperature for 1 hour. After washing, 100 µL of BluePhos substrate (Kirkegaard and Perry) was added, and the plates were incubated for 1 hour in the dark at room temperature. Color development after 30 minutes in the dark was determined at A₆₅₀ on a microplate reader (Versa Max, Molecular Devices, Sunnyvale,

CA) and analyzed with SoftMax® Pro 6 (version 6.5.1, Molecular Devices). The optical density (OD) value represents the value after subtraction of the negative control wells. ELISA OD values > 0.2 were used as a threshold to identify positive samples.¹⁶ ELISA controls included a negative peptide and canine and human sera that were negative by TRP19 ELISA and IFA to *E. canis*. Serum from a dog experimentally infected with *E. canis* (#2995) was used as a positive control, whereas the negative control was serum from a healthy laboratory-reared dog.²⁸ Human monocytic ehrlichiosis patient serum was also used as a positive control and normal human serum as a negative control. Similar ELISA assay conditions were used for *E. canis* genotype-specific TRP36 linear peptides: U.S. genotype (TEDSVSAPA), Brazilian genotype (ASVPEAE), and Costa Rican genotype (EASVPAEAPQPAQQTEFFSDGIEA). CI of 95% was calculated for the results of serum samples from naturally infected dogs by WinEpi 2.0 software free-accessible online (Working in Epidemiology, The University of Zaragoza, Zaragoza, Spain, available at: <http://www.winepi.net/>) using an ELISA test sensitivity and specificity of 100% for the TRP19 peptide.^{14,29}

Indirect immunofluorescence assay. Antigen slides were prepared from *E. canis*-infected DH82 cells, as described previously.²⁸ Human and dog sera were diluted (1:100) in PBS, and 10 µL was added to the antigen wells and incubated at 37°C for 30 minutes. The slides were washed with PBS, and 10 µL of the Alexa Fluor® 488 goat anti-human IgG or anti-dog IgG secondary antibody (1:100) (Invitrogen #A-11013 by Thermo Fisher Scientific, Waltham, MA) was added to the slides and incubated for 30 minutes. After washing, slides were observed using an Olympus BX61 epifluorescence microscope (Tokyo, Japan).

PCR. Genomic DNA (gDNA) was extracted from whole blood from 73 dogs that had TRP19 antibodies by ELISA using the DNeasy Blood and Tissue Kit (250) (69,506, QIAGEN Group, Hilden, Germany). These samples were amplified by PCR for *E. canis* 16S rRNA gene using primers ECA (5'-AACACATGCAAGTCGAACGGA-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3'), as previously described,³⁰⁻³² using 2.5 × 5 Prime Hot Master Mix (Quantabio, QIAGEN, Beverly, MA). *Ehrlichia canis* Jake strain gDNA was used as a positive control and molecular grade sterile water as a negative control. Selected samples were sequenced to determine identity of the PCR amplicon. Amplified PCR products were visualized by agarose gel (2%) electrophoresis containing 0.003% ethidium bromide carried out at 120 V for 40 minutes.

TABLE 1
Summary of blood, gDNA, and ticks obtained from dogs in Cauca municipalities

Municipality	Total dogs	Serum samples	Genomic DNA samples	Ticks from dogs*		
				<i>Amblyomma maculatum</i> s.l.†	<i>Amblyomma ovale</i>	<i>Rhipicephalus sanguineus</i> s.l.†
La Sierra						
Juana Castaña	4	4	2	2	0	4
El Tambo						
El Zarzal	16	16	9	0	0	43
El Placer	18	18	9	0	2	68
Betania	9	9	7	0	0	76
Santander de Quilichao						
Lomitas Arriba	17	17	12	0	0	150
Lomitas Abajo	23	23	17	0	0	88
Caloto						
Credo	19	19	11	0	0	6
Huellas	8	8	6	0	0	0
Total	114	114	73	2	2	435

* Tick specimens were primarily adult stage.

† s.l. = sensu lato.

TABLE 2
Serosurvey and PCR results from humans ($n = 506$) and dogs ($n = 114$) from four municipalities of Cauca (Colombia)

Municipality	TRP19 ELISA human samples	TRP19 ELISA dog samples	16S rRNA gene dog samples	<i>Ehrlichia canis</i> genotypes <i>trp36</i> gene		
				US	Br	CR
La Sierra						
Juana Castaña	0	4	1	0	0	0
El Tambo						
El Zarzal	0	9	2	2	0	0
El Placer	1*	9	6	1	0	5
Betania	0	7	3	2	1	0
Santander de Quilichao						
Lomitas Arriba	0	12	6	1	0	1
Lomitas Abajo	0	17	6	0	0	0
Caloto						
Credo	0	11	4	1	0	0
Huellas	0	6	2	2	0	0
Total	1	75	30	9	1	6

Br = Brazilian genotype (**ASVWPEAE**); CR = Costa Rican genotype (**EASVWPAEAPQPAQQTEDEFFSDGIEA**); US = U.S. genotype (**TEDSVSAPA**).

* Sample was negative by indirect immunofluorescence assay.

Genomic DNA from selected 16S rRNA PCR-positive samples ($n = 16$) was amplified by PCR for *E. canis trp36* DNA using the primers TRP36-F2 (5'-TTTAAACAAAATTAACACACTA-3') and TRP36-R1 (5'-AAGATTAACCTAATACTCAATATTACT-3') for the external reaction, and TRP36-D-F (5'-CACACTAAAATGTATAATAAAGC-3') and TRP36-R1 for the hemi-nested reaction.⁶ The PCR amplification was performed as described earlier with some differences: (95°C, 3 minutes), 30 cycles of denaturation (95°C, 30 seconds), annealing (45°C, 30 seconds), extension (70°C, 1 minute), and a final extension at 70°C for 5 minutes. The PCR amplification conditions were identical for the external and nested PCR reactions. PCR amplicons were purified using the MinElute[®] PCR purification kit (250) (28,006, QIAGEN). Then, they were sequenced at the Molecular Genomics Core of the University of Texas Medical Branch (Galveston, TX) using the Sanger method and analyzed by DNASTAR[®] Lasergene Genomics suite (version 14.1, DNASTAR Inc., Madison, WI) and SnapGene[®] Viewer (version 4.1.9, GSL Biotech LLC, Chicago, IL).

RESULTS

Humans and dogs in the study population. A total of 506 adults (> 18 years old) completed questionnaires and provided blood samples. A summary of the information of study participants is presented in Supplemental Table S2. Information obtained regarding the dogs and ticks included in this study is summarized in Table 1.

PCR. Genomic DNA samples ($n = 73$) were amplified by PCR targeting the *E. canis* 16S rRNA gene. Amplicons were detected in 30 (41%) samples by PCR. Selected samples ($n = 16$) were sequenced to identify the specific agent, and all samples had 100% homology to the 16S rRNA gene sequence of the *E. canis* YZ-1 strain (GenBank accession No. CP025749.1). To determine the *E. canis* genotype of these 16 samples, we amplified the *trp36* gene. We detected three different genotypes: United States ($n = 9$), Brazilian ($n = 1$), and Costa Rican ($n = 6$) (Table 2). All three *E. canis* TRP36 genotypes were detected by PCR in the sequenced samples of the El Tambo municipality. The GenBank

FIGURE 1. Immunoanalysis of 13 randomly selected Colombian dog sera showing correspondence between *E. canis* indirect immunofluorescence assay (IFA) and *E. canis* TRP19 ELISA (cutoff value, 0.2). *E. canis* = *Ehrlichia canis*.

accession numbers for each of the 16 *E. canis* *trp36* gene sequences are given in the Supplemental Table S3.

ELISA. Serosurvey results for the human and dog samples collected in four municipalities of Cauca are described in Table 2. All human samples were determined to be ELISA negative for anti-*E. canis* TRP19 peptide antibodies, except one sample (H181), which was negative by *E. canis* IFA. Over half (75/114; 66%; CI: 57.1–74.5%) of the sera collected from dogs had detectable antibodies to the *E. canis* TRP19 peptide. Selected TRP19 ELISA-positive ($n = 8$) and –negative ($n = 5$) samples were tested by IFA to confirm *E. canis* antibody status (Figure 1). Subsequently, a serologic genotypic (United States, Brazil, and Costa Rica) investigation of 16 selected dogs was carried out in which TRP36 gene sequences were obtained by PCR. Most dogs (14/16; 88%) had the highest antibody response to the TRP36 peptide that corresponded to the TRP36 genotype identified by TRP36 gene PCR/DNA sequencing (Figure 2). Half of these dogs ($n = 8$) had antibodies to more than one TRP36 peptide, suggesting sequential or coinfections had occurred. Data of *Rhipicephalus sanguineus* s.l. collection and *E. canis*

serological or molecular detection did not follow a normal distribution (Supplemental Table S4). Also, no significant association of cross-tab data between these variables was found (Supplemental Table S5).

DISCUSSION

In this study, the proportion of *E. canis* 16S rRNA PCR-positive dog samples was similar to a previously reported study in Colombia,²² but higher than that in a report from Medellín (Colombia).¹⁸ Notably, other studies in Colombia have reported a lower prevalence of *E. canis* in dogs (between 15% and 28%) using the *dsb* gene by real-time PCR.^{20,21} We unsuccessfully targeted the *E. canis* *dsb* gene in the Colombian dog samples by conventional PCR, but a single-step *E. canis* 16S rRNA gene PCR amplification provided increased sensitivity enabling detection of *E. canis* DNA. According to a meticulous review,³⁰ PCR protocols targeting the 16S rRNA gene were developed for identification of genera within the Anaplasmataceae family, requiring subsequent PCR assays for identifying a species. Thus, we sequenced half of the

FIGURE 2. Summary of 16 *Ehrlichia canis* *trp36* PCR-positive and TRP36 genotype-specific peptides ELISA (cutoff value, 0.2). Br = Brazilian genotype; CR = Costa Rican genotype; US = U.S. genotype.

positive samples to confirm the identity of the amplicon. In addition, we targeted the *E. canis trp36* gene for genotyping purposes and detected multiple *E. canis* genotypes, including the Costa Rican genotype, which has been detected in human blood bank donors in Costa Rica and in domestic dogs from Peru.^{6,17}

As previously reported in Colombia, the high *E. canis* seroprevalence we detected in Cauca dogs is similar to that reported in other studies in Colombia, ranging from 62% to 82%.^{19,20,22} In those studies, dogs were from a mixed group of owned, military, shelter, and/or even free-roaming dogs presented at veterinary clinics for examination. Notably, a recent Colombian study found a seroprevalence of around 30% in 300 dogs attending veterinary teaching hospitals in Medellín.¹⁸ Although we observed a higher seroprevalence, this difference is likely to be explained by geographic (rural versus urban) differences in the selected dog population and tick density.³³ In addition, the continuous and heavy exposure of dogs mainly to *R. sanguineus* sensu lato ticks in the endemic areas and a lack of adequate tick control programs may explain the high seroprevalence in these rural areas of Colombia.^{34,35}

The ELISA-based *E. canis*-specific TRP36 peptide seropositivity that we obtained is considered as evidence of active infection or previous exposure to *E. canis*. It is intriguing that the Colombian dogs infected with the Brazilian or Costa Rican genotypes showed no evidence of infection or exposure to the U.S. genotype. Also, none of the three *E. canis* genotypes were detected by ELISA at the same time in any sample. A previous research on 300 Colombian dogs from Medellín detected antibodies to the same three *E. canis* genotypes by ELISA in three dogs: one dog had the Costa Rican genotype and the remaining dogs had the U.S. genotype amplified by PCR.¹⁸ They found more dogs with the U.S. genotype, by serological and molecular tools, which is consistent with our results.

In our study, close contact of the surveyed individuals with tick-infested dogs was common. Such circumstance has been shown to increase the likelihood of finding ticks on humans in the U.S. east coast.³⁶ However, in the large human serosurvey we conducted, only one human sample (H181, an elderly woman) was ELISA positive to the *E. canis* TRP19 peptide, but negative by the IFA test with the *E. canis* Jake strain antigen. Nevertheless, there was little evidence of *E. canis* transmission to humans in the individuals surveyed in our study. Although humans can be parasitized by *R. sanguineus* s.l. when cohabiting with tick-infested dogs,³⁷ it is considered a rare event in countries such as Brazil where *R. sanguineus* is not frequently known to bite humans compared with other ixodid species in endemic areas.³⁸

In conclusion, our serological findings regarding *E. canis* TRP36 genotypes suggest that sequential or coinfections with multiple *E. canis* genotypes may be occurring, as has been reported previously.^{16,18} The TRP36 genotype detected by ELISA, in most cases, elicited the predominant serological response to the peptide representing the respective genotype. *Ehrlichia canis* antibodies were not detected in human sera ($n = 506$), but a majority (66%; $n = 114$) of dogs were seropositive. Three genotypes based on *E. canis trp36* gene were detected in Cauca dogs representing the U.S., Brazilian, and Costa Rican genotypes. This study suggests that although *E. canis* infection in dogs is highly prevalent in these

municipalities, there was no evidence of zoonotic transmission to humans. Moreover, considering the high seroprevalence, periodic epidemiological surveillance and effective control measures for the brown dog tick should be considered in these areas as a strategy to reduce *E. canis* infections in canines.

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