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Molecular and serological detection of *Trypanosoma cruzi* in dogs (*Canis lupus familiaris*) suggests potential transmission risk in areas of recent acute Chagas disease outbreaks in Colombia

Jeiczon Jaimes-Dueñez[a,b,*], Omar Triana-Chávez[b], Omar Cantillo-Barraza[b], Carolina Hernández[c], Juan David Ramírez[c], Agustín Góngora-Orjuela[a]

[a] Grupo de Investigación en Reproducción y Genética Animal GIRGA, Programa de Medicina Veterinaria y Zootecnia, Facultad de Ciencias Agropecuarias y Recursos Naturales, Universidad de Los Llanos, Villavicencio, Colombia
[b] Grupo de Biología y Control de Enfermedades Infecciosas BCEI, Programa de Biología, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Medellín, Colombia
[c] Grupo de Investigaciones Microbiológicas – UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia

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ABSTRACT

Chagas disease is a zoonotic infection widely distributed in tropical and subtropical regions of America, including more than 50% of the Colombian territory. In the last years, an increase of outbreaks of acute Chagas disease has been observed in the east of the country due to environmental changes and mammal movements toward human settlements. Given the importance of dogs (*Canis lupus familiaris*) as reservoir hosts and sentinels of *Trypanosoma cruzi* infection across different regions of America, in this study we reported a serological and molecular detection of *T. cruzi* infection in 242 dogs from an endemic area of Meta department (East of Colombia), with recent emergence of acute Chagas disease outbreaks. The distribution of *T. cruzi* infection in dogs was not homogeneous, ranging from 0–41.4% and 0–5.1% in different sampling sectors, through serological (ELISA/IFAT) and molecular methods (conventional and real time PCR), respectively. Statistical analysis indicated that dog infection was associated with specific sampling sectors. Our results show a moderate seroprevalence of infection and active circulation of *T. cruzi* in dogs from this zone, which suggest areas with potential risk of infection to human that must be taken into consideration when Chagas disease control programs need to be implemented.

1. Introduction

American trypanosomiasis or Chagas disease, a zoonosis caused by *Trypanosoma cruzi* affects more than 8 million people, resulting in approximately 12,000 deaths annually in Latin America (PAHO/WHO, 2016). *T. cruzi*, is a multi-host parasite subdivided into at least six Discrete Typing Units TcI – TcVI (DTUs) and one novel genotype named TcBat associated to anthropogenic bats (Marcili et al., 2009; Ramírez et al., 2014). Additionally, intra-genetic diversity has been reported within TcI suggesting the existence of two genotypes associated to transmission cycles (TcIDom and Sylvatic TcI) (Hernández et al., 2016a; Ramírez et al., 2012, 2013a). This kinetoplastid is adaptable to several mammalian species in diverse ecological niches and mainly transmitted by contact with feces/urine of infected blood-sucking triatomine bugs (vector transmission), blood transfusion, oral ingestion, laboratory accidents, organ transplantation and/or vertical transmission are other routes of transmission (Bezerra et al., 2014; Orozco et al., 2013; PAHO/WHO, 2016).

In Colombia, it is estimated that 0.96% of the population living in endemic areas for *T. cruzi* is infected, representing approximately 437.960 individuals (WHO, 2015). Vector transmission represents a public health problem where *Rhodnius prolixus* is present in domiciliary and extra-domiciliary ecotopes (Angulo and Esteban, 2011; Angulo-Silva et al., 2016; Guhl et al., 2007). In the last two years, unusual increases of acute outbreaks of Chagas disease of presumptive oral transmission have been reported in Colombia. Among the most relevant outbreaks, the outbreak in the municipality of Paz de Ariporo (Casanare) and Restrepo (Meta), which produced around forty acute cases of Chagas disease with mortality

* Corresponding author at: Grupo de Biología y Control de Enfermedades Infecciosas, Universidad de Antioquia, Calle 70 No. 52-21, Medellín, Antioquia, Colombia.
E-mail address: jeiczon05@gmail.com (J. Jaimes-Dueñez).

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rates up to 2.8%, lead to conclude that the intensive environmental changes that have taken place in this region have increased the likelihood of outbreaks emergence (Hernández et al., 2016c). Given the lack of knowledge about the transmission dynamics of *T. cruzi* in these municipalities and the proximity of mammal reservoirs to the outbreaks in urban areas, approaches to identify hotspots of transmission regions are necessary to prevent sporadic outbreaks.

In America, dogs (*Canis lupus familiaris*) are considered important sentinels hosts for *T. cruzi* infection in different countries such as Argentina, Venezuela, Panama and Colombia (Cantillo-Barraza et al., 2015; Crisante et al., 2006; Gurtler et al., 1996; Ramírez et al., 2013b; Saldaña et al., 2015). In addition, the epidemiological surveillance among these hosts has been pivotal for understanding the transmission dynamics and disease emergence in different endemic regions (Hernández et al., 2016c). Lastly, in different studies the screening of dogs has allowed to propose the synanthropic feature that this host may have connecting sylvatic and domestic foci (Calzada et al., 2015; Ramírez et al., 2013b). Therefore, the aim of this work was to conduct serological and molecular tests to determine *T. cruzi* infection in dogs from the urban area of the municipality of Cumaral – Meta, to unravel the importance of these hosts in outbreaks emergence.

2. Materials and methods

2.1. Study area and sampling

A transversal study was conducted between November and December 2015, in the municipality of Cumaral in the Meta department (neighboring municipality of Restrepo). Cumaral is located in the west of the Orinoco region, with latitude 4° 16′ 10″ N and longitude 73° 29′ 11″ W, at average altitude of 452 m (Fig. 1). The ecological zone consists of tropical rainforest surrounded by cattle pastures, crops and patches of native forest, with rainfall average of 3000 mm/year and an average annual biotemperature of 21 °C (IDEAM, 2014). The population is approximately 18,000 inhabitants and the economy depends mainly on agriculture, trade and tourism, with basic public services in 75.1% of houses (DANE, 2005). In the area, *T. cruzi* transmission is associated with high rates of *R. prolixus* infestation of palms next to dwellings as well as adaptation of this to human domiciles (Angulo et al., 2012).

Sampling was conducted in ten urban sectors (A–J) of Cumaral during a vaccination plan established by the zoonoses control Program (Fig. 1). The sectors were established to serve the canine population of this municipality, including two small villages (H, J), located at 13 and 17 km east of Cumaral, respectively. The sample size was calculated using Epi Info 7.0, taking into account a population of ~2300 dogs in the study area, a 15% of probability of being infected (according to Cantillo-Barraza et al., 2015), a 95% confidence interval and a statistical error of 5%. The estimated sample size was 180 but was increased in 30% to compensate sampling errors. Within each sector, the sample size was estimated according to the percentage of animals on each sector. Inclusion criteria for selected dogs were as follows: (i) born and raised in the study area, (ii) having a recognizable owner, and (iii) information available about the animal's history (i.e., age, breed, origin and general health). During the sampling, owner of each dog selected randomly received an informed consent to participate in the study.

2.2. Collection and processing of blood samples

For each animal, two blood samples of 5 mL were collected from radial vein, using serum and EDTA.K3 vacutainers, and stored at 4 °C until processed. For serum extraction, samples were centrifuged at 5000 × *g* by 10 min and serum was stored at −20 °C until serological assays were performed.

2.3. Serological diagnostic test

Detection of anti *T. cruzi* antibodies (IgG) was conducted using an Enzyme-Linked Immunosorbent Assay (ELISA) and an Indirect Immunofluorescence Antibody Test (IFAT). For both techniques, the antigen was prepared from harvested epimastigotes of the *T. cruzi* Colombian strains Cas-15 and Gal-61, previously characterized as TcI (Rojas et al., 2007). For ELISA, a whole lysate extracted from epimastigotes was used as antigen, while for IFAT it was obtained from complete epimastigotes fixed in formaldehyde 1% as reported elsewhere (Beltrán et al., 2001). The cut-off was determined as optical absorbance ≥0.200 (mean ± 3SD) for ELISA and sera dilution of 1/40 for IFAT as reported elsewhere (Cantillo-Barraza et al., 2015). Animals were defined as positive when samples were reactive to both tests, which have sensitivity 100% and specificity 98.7% respect to ELISA and IFAT from Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil (Xavier et al., 2012).

2.4. Molecular detection tests

To evaluate the role of dogs as *T. cruzi* reservoirs, molecular detection was carried out only in the positive animals by serology (ELISA/IFAT). Genomic DNA was extracted from 200 μl of blood with EDTA using the Genomic DNA Purification Kit (DNeasy Blood & Tissue Kit Qiagen®) following manufacturer's instructions. According to validation criteria for molecular detection of *T. cruzi* in Colombia, by each animal a conventional PCR (cPCR) and quantitative real time PCR (qPCR) for detection of satellite DNA and an internal amplification control (IAC) were performed as reported elsewhere (Hernández et al., 2016a; Ramírez et al., 2015, 2009). The qPCR test was considered positive when the amplification exceeded the threshold of fluorescence 0.01, and in cPCR when was observed a DNA fragment of 166 bp in the electrophoresis. Both molecular tests have specificity of 100% and analytical sensitivity of 0.70 parasite equivalents/mL (Ramírez et al., 2015). Finally, to confirm *T. cruzi* infection and identify co-infections with *T. rangeli*, positive samples for satellite nuclear PCR (qPCR and cPCR) were confirmed by cPCR based on kinetoplast minicircle of *T. cruzi*, using published primers and conditions (Fitzwater et al., 2008; Sturm et al., 1989; Ramírez et al., 2009). A positive result was considered based on the appearance of the characteristic 330-bp product for *T. cruzi*, and two products of 300 and 450-pb for *T. rangeli*. PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and detected by UV light.

2.5. DTUs discrimination

In order to detect the six DTUs and the two subdivisions of TcI (TcIDom and sylvatic TcI) in the *T. cruzi* positive samples, PCR was performed using the intergenic region of the mini-exon gene, the 24Sa, 18S and A10 regions as reported elsewhere (Hernández et al., 2016a; Villa et al., 2013).

2.6. Statistical analysis

To evaluate the pattern of statistical relationships between *T. cruzi* diagnosis (positive PCR or IgG presence) and epidemiological variables (age, sex, breed size and sampling sector) (Table 1), a multiple correspondence analysis (MCA) was run using XLSTAT v.2017. MCA is a multivariate analysis which allows investigating the pattern of relationships between two or more categorical variables using geometrical methods by locating each variable/unit of analysis as a point in a low-dimensional space (Tian et al., 1993).

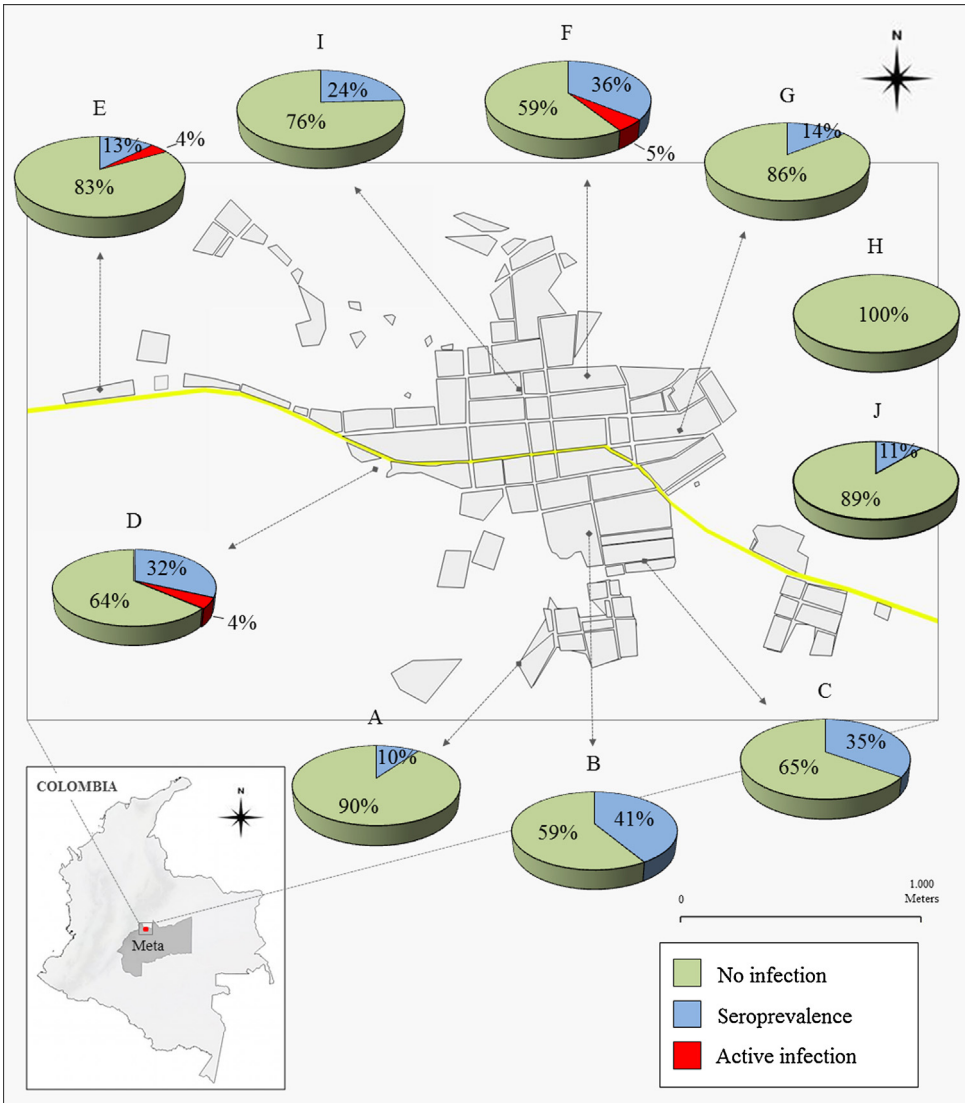

Fig. 1. Study area and *T. cruzi* infection in dogs from the sampling sectors.

To select clusters of categories significant in each dimension, value-test with ($P < 0.05$) was considered. Only variables with *eigenvalues* ≥0.90 were kept in the analysis as reported elsewhere (Goldberg and Chamblee, 1997).

2.7. Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the Colombian code of practice for the care and use of animals for scientific purposes, established by Law 84 of 1989. Ethical approval (Act N° 04 of 2015) for analyzing animal specimens was obtained from the animal ethics Committee of the University of the Llanos, Villavicencio, Colombia.

3. Results

A total of 242 dogs were examined, which corresponded to 48.7% of females and 78.9% of animals of creole breed. The mean and standard deviation age was 2.9 ± 2.6 years (range from 3 months to 15 years). No animals manifested significant alterations in its general health.

3.1. Serological detection of T. cruzi infection

Sixty-two dogs (25.6%) were positive for *T. cruzi* by both ELISA and IFAT tests. Prevalence of infection among sector varied from 0.0% to 41.4% in the sectors H and B, respectively (Table 1 and Fig. 1). Regarding to other variables, higher rates of infection were observed in the categories of geriatrics (36.4%), female (27.1%) and large breeds (34.5%) (Table 1). Statistical analysis showed *eigenvalues* ≥0.90, only for serological diagnosis, age, sex, breed size and sampling sector, obtaining a summary model that explains 55.2% of the variability, and suggest a significant cluster between animals positive by serology and sampling sectors B, C and F ($P < 0.05$) (Fig. 2).

3.2. Molecular detection and genotyping of T. cruzi infection

A total of four dogs (1.6%) amplified successfully *T. cruzi* DNA satellite nuclear (qPCR and cPCR), and were confirmed by cPCR for kinetoplast DNA minicircle. No co-infection with *T. rangeli* was observed. Positive animals were detected only in the sectors D, E and F with prevalences of 4.5%, 4.2% and 5.1%, respectively (Table 1 and Fig. 1); and the age groups of puppy and young animals with prevalences of 2.0% and 2.5%, respectively (Table 1). Genotyping

Table 1
Prevalence of *T. cruzi* infection in dogs from Cumaral – Meta, Colombia.

Independent variables	Animals screened	*Trypanosoma cruzi*			
		(ELISA + IFAT)		(qPCR + cPCR)	
		Positive	Prevalence (%)	Positive	Prevalence (%)
Age group					
Puppy (3 m to <1 y)	50	11	22.0	1	2.0
Young (≥1 y to <4 y)	120	29	24.1	3	2.5
Adult (≥4 y to 7 y)	50	14	28.0	0	0.0
Geriatric (≥7 y)	22	8	36.4	0	0.0
Sex					
Female	118	32	27.1	1	0.8
Male	124	30	24.2	3	2.4
Breed size					
Large	29	10	34.5	0	0.0
Medium	191	48	25.1	4	2.1
Small	22	4	18.2	0	0.0
Sampling sector					
A	30	3	10.0	0	0.0
B	29	12	41.4	0	0.0
C	20	7	35.0	0	0.0
D	22	8	36.4	1	4.5
E	24	4	16.7	1	4.2
F	39	16	41.0	2	5.1
G	14	2	14.3	0	0.0
H	13	0	0.0	0	0.0
I	33	8	24.2	0	0.0
J	18	2	11.1	0	0.0

Notation: m = months; y = years; No. = number; df = degrees of freedom.

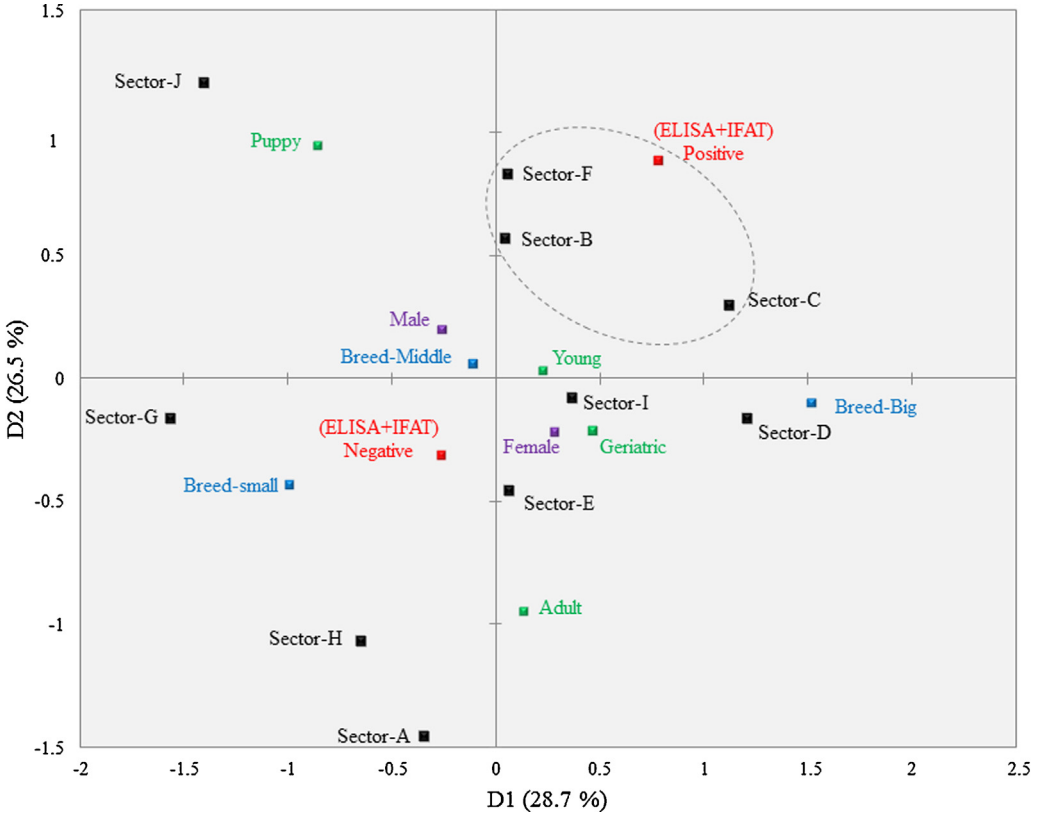

Fig. 2. Multiple correspondence analysis of association between serological diagnoses of *T. cruzi*, age, sex, breeds size and sampling sector.

revealed sylvatic TcI DTU in a dog of the sector D while the other samples did not amplify efficiently for all markers. Statistical analysis showed an *eigenvalue* <0.90 for this variable (active infection).

4. Discussion

In Colombia, environmental changes such as deforestation and global warming have affected ecotypes and the behavior of *T. cruzi* vectors and reservoirs so that these have become displaced to new

areas, thereby leading to new transmission scenarios that favor outbreaks appearance (Hernández et al., 2016c; Rueda et al., 2014). Given the increase of this trend in the Orinoco region, prevention and control measures for Chagas diseases are necessary to reduce these events. Herein, we addressed a *T. cruzi* serological and molecular surveillance in dogs from an urban area of Cumaral – Meta, where previous outbreaks of Chagas disease have been reported (Hernández et al., 2016c). A moderate seroprevalence of infection and areas with active circulation of the parasite was observed, suggesting active transmission in urban areas and a considerable role of dogs as reservoirs of *T. cruzi* in this municipality.

In this study, we observed that seroprevalence in dogs from Cumaral is higher than that detected in localities such as Boyacá and Magdalena departments (10.7–15.0%) where significant infestation rates of *R. prolixus*, *T. dimidiata* and *Rhodnius pallescens* have been found (Turriago et al., 2008; Vásquez et al., 2013); but comparatively lower than the values detected in localities of Bolivar department (70.1%), where high *Triatoma maculata* infestation rate in peridomestic areas could explain this behavior (Cantillo-Barraza et al., 2015). These data suggest that *T. cruzi* infection rates in Colombian dogs could be modulated by the dynamics of vector populations, which are considered the main source of infection (Gurtler et al., 1996; Gürtler et al., 1993). In Cumaral, at least three species of triatomine bugs have been reported including *Panstrongylus geniculatus*, *R. prolixus* and *R. pictipes* with abundances of 38.1%, 36.3% and 25.4% in the positive homes, respectively (unpublished data). This behavior combined with the *T. cruzi* infection rates of 40.0%, 28.0% and 4.7% observed in Colombian populations of these species, respectively (Hernández et al., 2016b), suggest that *P. geniculatus* is the main species that modulate *T. cruzi* infection in dogs of this municipality. However, studies of food sources and dynamic population of these insects must be conducted to confirm this premise.

Regarding the active infection rate of *T. cruzi*, the values reported by us are similar to those observed in dogs from enzootic areas of Boyacá (2.1%) through xenodiagnosis tests (Turriago et al., 2008), but comparatively lower than observed in the outbreak from Restrepo – Meta (27.5%) using molecular techniques (Hernández et al., 2016c). The outbreak study in Restrepo was carried out in a rural area with high abundance of wild reservoir (opossums) and insects (*P. geniculatus* and *R. pictipes*) infected, and parasitemias with TcIV in 50% of the positive dogs (Hernández et al., 2016c). These findings suggest that differences observed could be due to ecological conditions and DTUs specific circulating that favored a high active infection rate in the outbreak area, and highlights the importance of molecular studies in dogs to evaluate the *T. cruzi* dynamics of transmission in different scenarios of endemic areas. Finally, the molecular tests carried out only in the seropositive animals, also could explain the low active infection rate, thus molecular screening in all samples is recommended for future studies.

On the other hand, the active parasitemia of *T. cruzi* in dogs is modulated by host factors (stress, body condition and polyparasitism), ecological conditions (continue exposure to infected bugs, wild reservoirs and ecological changes) and parasite traits as DTUs (Cantillo-Barraza et al., 2015; Enriquez et al., 2014, 2016). In this study, we observed that moderate seroprevalence of *T. cruzi* in dogs from Cumaral is not proportional with the active infection rate (Table 1 and Fig. 1). Similar results have been observed in Brazil, where despite high seroprevalence in dogs, no high active infection rate has been observed in these animals (Tavares et al., 2015; Xavier et al., 2012), but differ from those observed in Argentina where a high seroprevalence and high active infection rates has been evidenced (Enriquez et al., 2013; Monje-Rumi et al., 2015). This behavior has been associated with the domestic transmission cycle of TcI in dogs from Brazil, and TcV/TcVI in dogs from Argentina, suggesting that infection with TcI in dogs produces considerable parasitemia during the acute phase of infection, but it is negligible during the chronic phase (Tavares et al., 2015; Xavier et al., 2012). In this study, the circulation of sylvatic TcI was detected in one positive dog, suggesting that moderate seroprevalence and low active infection rate detected in this population may be due to transmission cycle of sylvatic TcI in these animals. Additionally, the association of sylvatic TcI to recent Chagas disease outbreaks in the Orinoco region (Hernández et al., 2016c; León et al., 2015), and the detection of this DTU in animals with chronic infection (IgG positive), suggests an important role of dogs from this zone as synanthropic reservoirs of *T. cruzi* that could establish a link between the sylvatic and domestic transmission cycles, as demonstrated in other regions of Colombia (Ramírez et al., 2013b).

Although this study was performed in an urban area and outside of the outbreak period, our results showed that 75% of dogs with active parasitemia were ≤1 year old (Table 1), and one of them corresponded to a puppy with five months old, evidencing recent infections in this group of animals, which suggest hotspot transmission and areas with potential risk of infection for people. According to studies in Argentina, infected dogs in a household may quadruple the risk of infection in infants (Basombrio et al., 1993), suggesting that people living in the sectors D, E and F (sectors with active parasitemia), might have had more risk of infection compared with people living in other sectors (Table 1 and Fig. 1).

Finally, statistical analysis showed a significant association between positive animals by serology and sampling sector B, C and F (Fig. 2). These results are comparable to those observed in localities of Bolivar department where no significant differences in the seroprevalence of *T. cruzi* were found according to dogs' sex, age and other variables evaluated (Cantillo-Barraza et al., 2015), indicating that *T. cruzi* infection in dogs is mainly modulated by spatial conditions. In America, *T. cruzi* seroprevalence in dogs has been associated with their proximity to forest, selective abundance of small wild mammals and economic status of the human communities (Xavier et al., 2012; Fung et al., 2014). Although, we did not evaluate these variables in our study, we consider that specific conditions in the sectors B, C and F, may favor the abundance of synanthropic reservoirs and insects facilitating the *T. cruzi* transmission to dogs from these places, as evidenced in sector F, which besides having a high seroprevalence had the highest active infection rates (Fig. 1). However, we did not find active parasitemia in sectors B and C that could suggest temporal changes in its ecological conditions or enzootic stability of *T. cruzi* infection across these sectors. Therefore, longitudinal studies of *T. cruzi* infection in dogs that include other variables such as ecological and socioeconomic features of these sectors must be conducted to confirm these hypotheses.

In conclusion, the serological and molecular surveys of *T. cruzi* in dogs from Cumaral – Meta during vaccination plan, allowed establishment of the prevalence and distribution of infection of *T. cruzi* in this zone, suggesting areas with potential risk of transmission to humans, which must be taken into consideration when Chagas disease control programs are implemented in this municipality.

Conflict of interest statement

All the authors have participated in the study and no conflicts of interests have been disclosed.

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