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Trypanosoma cruzi diversity in the Gran Chaco: Mixed infections and differential host distribution of TcV and TcVI

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ABSTRACT

The transmission cycles of *Trypanosoma cruzi* in the Gran Chaco are complex networks involving domestic and wild components, whose interrelationships are not well understood. Knowing the circuit of transmission of the different Discrete Typing Units (DTUs) of *T. cruzi* in the complex environment of the Chaco region is relevant to understanding how the different components (reservoirs, vectors, ecotopes) interact. In the present study we identified the DTUs infecting humans and dogs in two rural areas of the Gran Chaco in Argentina, using molecular methods which avoid parasite culture. Blood samples of humans and dogs were typified by PCR-DNA blotting and hybridization assays with five specific DNA probes (TcI, TcII, TcIII, TcV and TcVI). PCR analyses were performed on seropositive human and dog samples and showed the presence of *T. cruzi* DNA in 41.7% (98/235) and 53% (35/66) samples, respectively. The identification of infective DTUs was determined in 83.6% (82/98) and 91.4% (32/35) in human and dog samples, respectively. Single infections (36.7% – 36/98) and a previously not detected high proportion of mixed infections (47.9% – 47/98) were found. In a 15.3% (15/98) of samples the infecting DTU was not identified. Among the single infections TcV was the most prevalent DTU (30.6% – 30/98) in human samples; while TcVI (42.8% – 15/35) showed the highest prevalence in dog samples. TcV/TcVI was the most prevalent mixed infection in humans (32.6% – 32/98); and TcI/TcVI (14.3% – 5/35) in dogs. Significant associations between TcV with humans and TcVI with dogs were detected. For the first time, the presence of TcIII was detected in humans from this region. The occurrence of one human infected whit TcIII (a principally wild DTU) could be suggested the emergence of this, in domestic cycles in the Gran Chaco.

1. Introduction

Chagas disease is an endemic antropozoonosis in Latin America, where about 7–8 million people are affected (WHO, 2014). The illness is caused by the protozoan parasite *Trypanosoma cruzi*, which presents a broad intraspecific genetic diversity, as indicated by its subdivision into six Discrete Typing Units (DTUs), TcI–TcVI (Zingales et al., 2009). The diverse DTUs are differentially distributed across geographical areas in Latin America and maintained in domestic and wild cycles of transmission alternating between vector species and mammalian hosts (Zingales et al., 2012). TcI is widely dispersed throughout America and is associated with both, domestic and wild transmission cycles. TcIII and TcIV appear mostly in sylvatic cycles, while TcII, TcV and TcVI in the domestic cycle (Miles et al., 2009).

In the Southern cone of America, the Gran Chaco is a hyperendemic region for Chagas disease that stretches over Argentina, Bolivia and Paraguay where domestic and wild transmission cycles occur (Gürtler, 2009). Some studies in this region have shown complex eco-epidemiological scenarios, with diverse seroprevalence

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and infestation rates, and different *T. cruzi* DTUs flowing between domestic and sylvatic cycles (Brenière et al., 2002; Diosque et al., 2003, 2004; Cardinal et al., 2008; Moreno et al., 2012; Perez et al., 2013). In rural communities from northern Argentina, the domestic cycle involves humans, domestic and synanthropic animals, and domiciled *Triatoma infestans* (Gürtler, 2009). Particularly, in domestic cycle of north-western Argentina, TcI, TcIII, TcV and TcVI were identified in humans, dogs, cats and *T. infestans* by mean of classical methods of parasite isolation (xenodiagnoses and blood culture) (Diosque et al., 2003; Cardinal et al., 2008;Tomasini et al., 2011; Maffey et al., 2012; Lauthier et al., 2012; Enriquez et al., 2013).

However, it is important to note that a selective process could be occurring when parasites are isolated using xenodiagnostic and blood cultures (Diosque et al., 2003; Macedo et al., 2004). These parasite selection may bias the frequency values of each DTU and mixed infections may be underestimated. In this sense, a direct characterization of parasite populations obtained from blood samples is of particular relevance. In this study, hybridization assays with specific probes from minicircle hypervariable regions (mHVRs) were used to assess the prevalence of the different *T. cruzi* DTUs in human and dog blood samples from two neighbour settlements located in the 12 de Octubre department, in Chaco Province, Argentina.

2. Materials and methods

2.1. Study area

The study was carried out in Las Leonas (27° 01′ 49″S; 61° 39′ 8.7″W) and El Palmar (27° 4′ 32,7″S; 61° 34′ 19,9″W), two neighbour settlements located in the 12 de Octubre department, Chaco Province (Argentina).

2.2. Ethical approval and informed consent

The protocol was approved by the Bioethics Committee of the Faculty of Health Sciences of the National University of Salta, Argentina. The objectives, scope and importance of participation in the study were explained to the community and all participants signed an informed consent form.

2.3. Study design and samples

We performed a cross-sectional study during March 2008 in Las Leonas and in February 2010 in El Palmar. A total of 154 dwellings were examined: 62/65 (95.4%) from Las Leonas and 92/99 (92.9%) from El Palmar. Biological samples and epidemiological data (inhabitants per house, sex, and age) were collected in each dwelling.

A total of 538 individuals voluntarily agreed to participate in this study (230 persons from Las Leonas and 308 from El Palmar).

A total of 323 dogs (142 dogs from Las Leonas and 181 dogs from El Palmar) were analyzed to determine infection by *T. cruzi*.

2.4. Human and dog samples

For *T. cruzi* diagnosis, seven millilitres of peripheral blood were obtained by venous puncture. Two millilitres were used for serum extraction and further stored at 4 °C. Five millilitres of blood were mixed with an equal volume of a solution of Guanidine 6 M-HCl and 0.2 M EDTA. The obtained samples were incubated in a water bath at 98 °C for 10 minutes and stored at room temperature until used (Britto et al., 1995).

2.5. Diagnosis of T. cruzi infections in humans and dogs

Humans serum samples were analyzed by Indirect Hemagglutination test (IHA, Chagatest HAI, Wiener Laboratory, Rosario, Argentina), Enzyme-Linked Immunosorbent Assay (ELISA, Chagatest ELISA recombinant, Wiener Laboratory, Rosario, Argentina) according to manufacturer instructions. Samples showing discordant results between ELISA and IHA were studied by Indirect Immunofluorescence assays (IIF), as described by Rumi et al. (2013). Individuals whose serum samples showed two positive serological tests were considered seropositive. Serum samples from dogs were analyzed by ELISA-*T. cruzi* homogenate according to the protocol described by Cimino et al. (2011).

2.6. Polymerase chain reaction and DTU identification

Blood samples from seropositive humans and dogs from both settlements were analyzed by PCR for detection of *T. cruzi* kDNA. DNA was extracted from 200 μl of the Guanidine HCl-EDTA/blood mixture with a standard phenol-chloroform protocol. Each sample was tested in duplicate and appropriate controls were used to rule out possible DNA contamination. PCR was carried out in a total volume of 50 μl reaction mix containing: 1X GoTaq amplification buffer, 3 mM $MgCl_2$ solution, 0.2 mM dNTP, 1.25 U of GoTaq DNA polymerase (Promega, USA), 0.5 μM of kDNA specific primer 121 (5′-AAATAATGTACGGG(T/G)GAGATGCATGA-3′) and 122 (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′) and 7.5 μl of template DNA. Amplification was performed in a MJR PTC-100 thermocycler (MJ Research, Watertown, MA, USA). Cycling parameters were: one step of 3 min at 94 °C; two cycles at 97.5 °C for 1 min and 64 °C for 3 min, followed by 33 cycles of 1 min at 94 °C followed by 1 min at 64 °C with a final extension at 72 °C for 10 min. Ten microliters of the PCR reaction were visualized in a 2% agarose gel stained with ethidium bromide.

2.7. T. cruzi DTU identification

DTU identification was carried out by hybridization with specific mHVR-kDNA non-radioactive probes from *T. cruzi* clones: X10cl1 (TcI), Tu18cl2 (TcII), M5631cl5 (TcIII), LL055R3cl2 (TcV) and CL-Brener (TcVI).

Briefly, Southern blot analysis was performed with 10 μl of each PCR product. Samples were subjected to electrophoresis, transferred to Hybond N + nylon membranes (Roche Diagnostics) and cross-linked using u.v. light to fix the DNA. The membranes were pre-hybridized for 30 min at 42 °C and hybridized with a panel of five genotype-specific probes labeled by the random priming method with digoxigenin-dUTP (Roche Diagnostics). After hybridization, the membranes were submitted to low and high stringency washings according to the manufacturer's instructions (Roche Diagnostics). Construction of specific probes was performed by amplification of the variable region of the *T. cruzi* minicircles. The primers for probe generation were CV1 (5′-GATTGGGGTTGGAG TACTAT-3′) and CV2 (5′ TTGAACGGCCCTCCGAAAAC-3′) which produced a 290-bp fragment. Restriction sites for Sau96I and ScaI which allow elimination of the minicircle constant region of these PCR fragments were included in the sequence of these primers (Veas et al., 1991). The CV1-CV2 PCR fragments were further digested with the restriction endonucleases obtaining a 250-bp final product. For validating our experimental conditions, each generated DNA probe was evaluated by Southern blot analysis against different 330-bp mHVR PCR products of several *T. cruzi* stocks. A total of 27 *T. cruzi* stocks previously characterized by Multilocus Enzyme Electrophoresis, (MLEE) and Multilocus Sequence Typing (MLST) as DTUs TcI (9 stocks), TcII (2 stocks), TcIII (3 stocks), TcV (7 stocks) and TcVI (6), from vectors and hosts of our

study area and humans from the province of Salta; and 7 reference strains (two reference strains for TcI and one reference strain for each of the remaining DTUs) were previously used to evaluate specificity of each probe. All of the probes were specific to their corresponding DTU as they cross-hybridized only with the homologous samples.

2.8. Data analysis

Seropositive rates were calculated as follow [No. of host (humans or dogs) seropositive/No. total of host (humans or dogs) studied]. Frequency of each DTU [No. of host (humans or dogs) infected by a determined DTU/No. total of host (humans and dogs) with positive PCR]. The χ^2 test was used in order to compare DTUs distribution in humans and dogs; χ^2 Yates correction was applied when at least one of the expected values was <5. The test of proportions was used in order to compare data from dwellings where both, TcV and TcVI were present. A p-value <0.05 was considered significant. All analyses were performed using the software Infostat (Di Rienzo et al., 2009) GraphPad (Prism version 5.0, GraphPad Software, San Diego California USA) and G*Power 3 (Faul et al., 2007).

3. Results

3.1. T. cruzi detection by serology and PCR in humans and dogs

The serum samples of 538 humans and 323 dogs from two settlements were analyzed. The median age of individuals was 29.5 years (IQR, 10–49) and 29.6 years (IQR, 12–45) from Las Leonas and El Palmar, respectively. Serological outcome and *T. cruzi* kDNA detection in humans and dogs from the two settlements are summarized in Table 1.

3.2. DTU identification

Amplicons from PCR positive samples were used as target to determine the infective *T. cruzi* DTU circulating in blood by using PCR-DNA blotting and hybridizations assays with five specific DNA probes (TcI, TcII, TcIII, TcV and TcVI). Infecting DTUs were identified in 84.6% (83/98) of samples from PCR positive humans; and 91.4% (32/35) of samples from PCR positive dogs. Fig. 1 summarizes the results of the obtained characterizations in samples of humans and dogs from both settlements.

In human samples, single infections were detected in 36.7% (36/98), whereas 47.9% (47/98) were mixed infections. No hybridization was detected in 15.3% (15/98) of positive PCR samples. Considering only single infections, TcV was the most frequency DTU in human samples (30.6% – 30/98); whereas TcV/TcVI was the most frequency mixed infection detected in 33% (32/98) of the human samples. As shown in Fig. 1, one mixed infection with TcIII/TcV (1% – 1/98) was observed. Fig. 2 shows the representative pattern of hybridization for human samples.

Results of hybridization with dog samples show single infections in 51.4% (18/35) samples and in 40% (14/35) mixed infections. A low percentage of samples (8.6% – 3/35) was not recognized by any used probe. TcVI was the most frequency DTU found in single infection in dog samples (42.8% – 15/35); whereas

Table 1
Serological outcome and detection of *T. cruzi* kDNA in human and dog samples from Las Leonas and El Palmar.

Settlements	No. of examined		Seropositive % (No. of positives)		PCR positives % (No. of PCR +)	
	Humans	Dogs	Humans	Dogs	Humans	Dogs
Las Leonas	230	142	40 (92)	11.3 (16)	35 (32)	75 (12)
El Palmar	308	181	47.1 (145)	27.6 (50)	45.5 (66)	46 (23)
Total	538	323	44.1 (237)	20.4 (66)	41.3 (98)	53 (35)

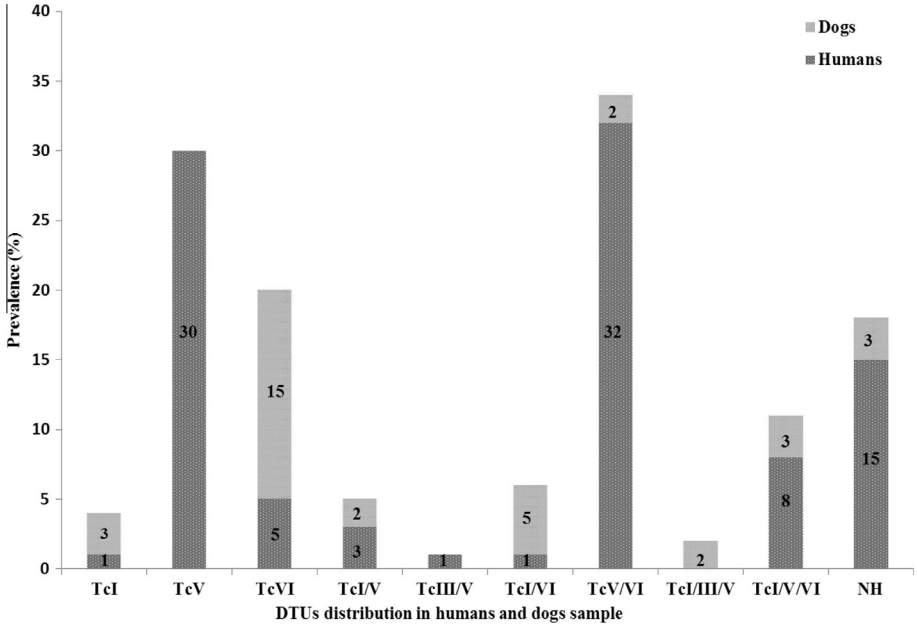

Fig. 1. DTUs distribution in blood samples for humans and dogs from two rural areas in Argentina Northwest.

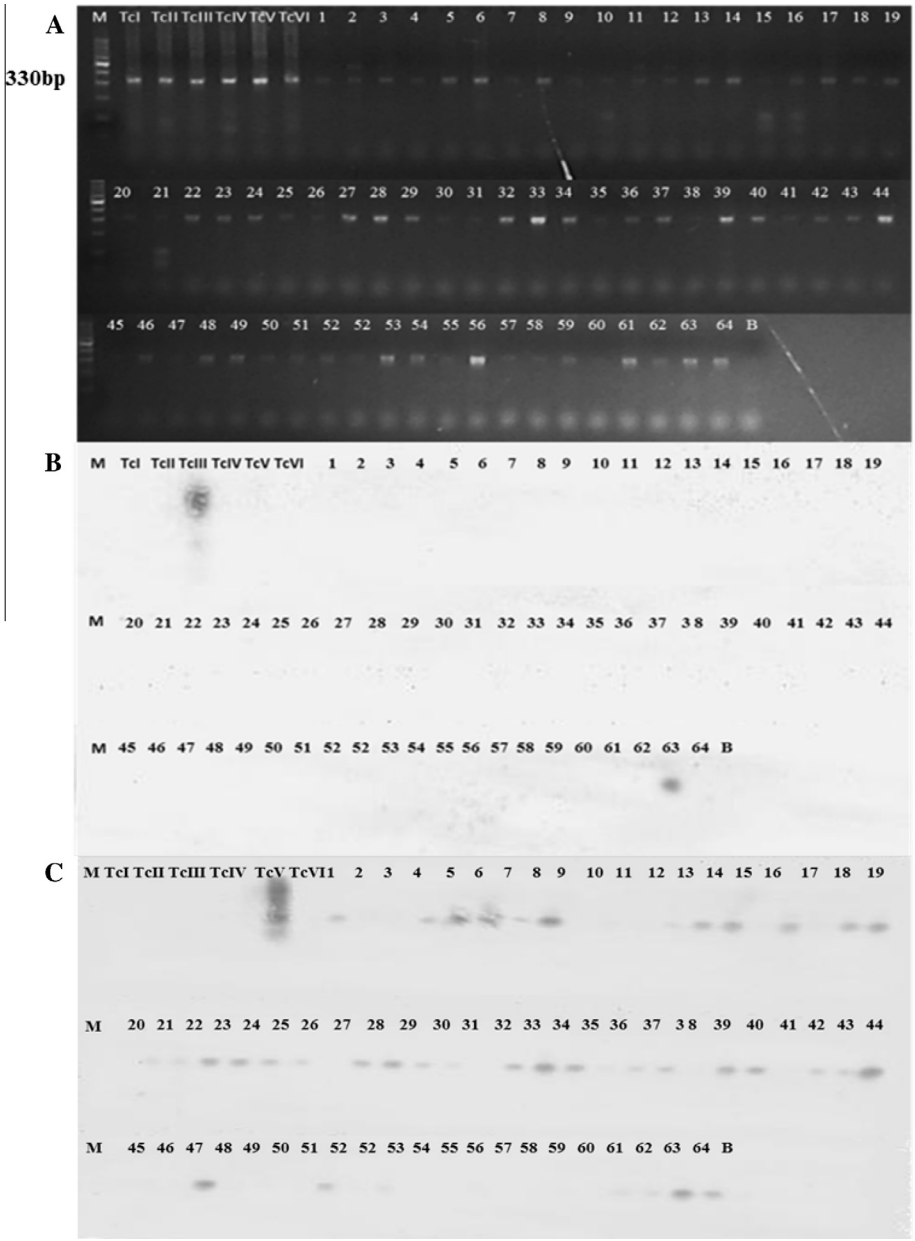

Fig. 2. (A) Representative electrophoretic pattern of minicircle regions of 300-bp amplified PCR for human blood samples. M, molecular weight marker (100-bp ladder; Promega); Lanes I–VI: positive control probe: TcI (X10cl1), TcII (Tu18cl2), TcIII (M5631cl5), TcIV (CANIIIcl1) TcV (LL055R3cl2) and TcVI (CL-Brener). Lanes 1–64: DNA from the blood samples of human. B: Blank. (B) kDNA of (A) transferred to a nylon membrane, hybridized with specific labeled probe against TcIII (M5631cl5). (C) kDNA of (A) transferred to a nylon membrane and hybridized with specific labeled probe against TcV (LL055R3cl2).

TcI/TcVI was the most frequency mixed infection in dogs (14.3% – 5/35). Fig. 3 shows the representative pattern of hybridization for dog samples.

3.3. Distribution of DTUs in humans and dogs

When only single infections were considered, statistically significant differences in the distribution of TcV and TcVI DTUs were detected (X^2 corrected = 28.67, df = 1, p = 0.001). TcV (30/98, 30.6%) was over-represented in humans and TcVI (15/35, 42.9%) was over-represented in dogs. These differential distributions of TcV and TcVI in humans and dogs were also observed when both, single and mixed infections were taken together: TcV was the most frequent DTU in human samples (76/98, 77.5%); while TcVI was the most frequent DTU in dog samples (25/35, 71.4%). These differential distribution of TcV and TcVI in humans and dogs was also statistically significant (X^2 = 13.02, df = 1, p = 0.003).

In order to examine if the observed associations (TcV/humans and TcVI/dogs) still remain significant at dwelling scale, we perform a test of proportions using data from dwellings where both, TcV and TcVI were present either, alone (single infection) or with other DTUs in the same host (mixed infection) (Table 2). The obtained results show that the association between TcV and humans was highly significant (p = 0.0001; Power test = 0.94), while TcVI/dogs association was not significant (p = 0.96; Power test = 0.06) at the dwelling scale.

4. Discussion

The transmission cycles of *T. cruzi* in the Gran Chaco are complex networks involving domestic and wild components, whose

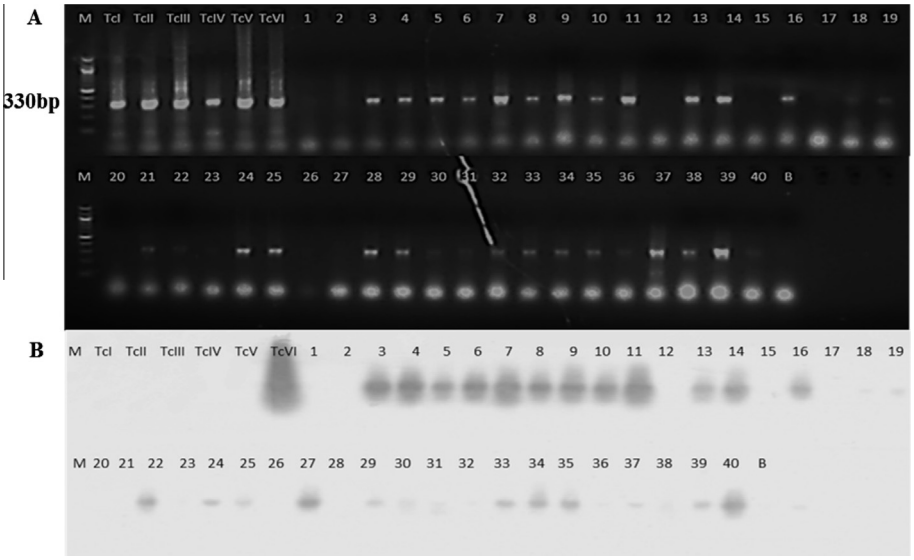

Fig. 3. (A) Representative electrophoretic pattern of minicircle regions of 300-bp amplified PCR for dog blood samples. M, molecular weight marker (100-bpladder; Promega); Lanes I–VI: positive control probe: TcI (X10cl1), TcII (Tu18cl2), TcIII (M5631cl5), TcIV (CANIIIcl1) TcV (LL055R3cl2) and TcVI (CL-Brener). Lanes 1–40: DNA from dog blood samples. B: Blank. (B) kDNA of (A) transferred to a nylon membrane and hybridized with specific labeled probe against TcVI (CL-Brener).

Table 2
Distribution of DTUs in humans and dogs at dwelling scale.

Area	House code	Humans		Dogs	
		DTUs	No. of individuals	DTUs	No. of individuals
Las Leonas	40	TcV/VI	1	TcI/VI	1
	41	TcV/VI	1	TcVI	2
		TcI/V/VI	2		
	44	TcI/V	1	TcVI	2
				TcI/V/VI	1
	51	TcI/V/VI	1	TcVI	2
El Palmar	4	TcV	3	TcI	1
		TcV/VI	1	TcI/VI	1
	9	TcV	2	TcVI	1
		TcV/VI	2	TcI/III/V	1
	16	TcV/VI	3	TcV/VI	1
	20	TcV/VI	4	TcI/V	1
				TcI/V/VI	1
	21	TcI/V/VI	1	TcI	1
	25	TcV/VI	1	TcI/V	1
	74	TcV	1	TcV/VI	1

interrelationships are not yet well understood. Knowing the circuit of transmission of the different DTUs in the complex environment of the Chaco region is relevant to understanding how the different components (reservoirs, vectors, ecotopes) interact. In this region, occurrence of the main *T. cruzi* DTUs (except TcIV and Tcbat) has been reported (Diosque et al., 2003; Cardinal et al., 2008; Tomasini et al., 2011; Lauthier et al., 2012; Maffey et al., 2012; Enriquez et al., 2013; Ragone et al., 2012). Moreover, probable associations among some DTUs and mammal hosts have been suggested. Specifically, significant higher frequencies of TcV in humans and TcVI in dogs have been observed (Diosque et al., 2003; Cardinal et al., 2008).

In the present work we study the frequencies of the different DTUs in humans and dogs (the main domestic reservoir of *T. cruzi* in the region) using a molecular approach and avoiding possible bias in the frequencies of the DTUs due to strain selection during culture. The results showed the presence of TcI, TcIII, TcV and TcVI in the studied hosts. A high frequency of TcV in humans and TcVI in dogs was observed. It is difficult to hypothesize the causes which explain the differential frequencies of TcV and TcVI in humans and dogs, since there are a high number of possible intervening variables. Taking into account that TcV and TVI are closely related from a genetic point of view, we suggest that no intrinsic characteristics of these DTUs would explain these associations. Indeed, when we examined if the associations between TcV/humans and TcVI/dogs were still observed at dwelling level, in houses where TcV and TcVI were present, we found that only association between TcV and humans, but not TcVI/dogs, since a similar proportion was observed for both host.

Detection of mixed infections was higher than the obtained in previous studies (Diosque et al., 2003; Cardinal et al., 2008; Enriquez et al., 2013), possibly due to both, technique sensitivity and absence of selection pressure in culture. In fact, we detected a total of 60 mixed infections over a total of 133 examined hosts (45.1%). Our results support the hypothesis that, in classical methods of parasite isolation (xenodiagnoses and blood culture), a selection process of DTUs could occur, and demonstrate that the direct identification of *T. cruzi* populations is necessary in order

to avoid selection bias and to better understand the transmission dynamics for different DTUs (Bosseno et al., 2000).

Regarding TcII, this DTU has been reported (in low frequency) in Argentinian patients with chronic Chagas disease (Cura et al., 2012) and congenital case (Burgos et al., 2007; Diez et al., 2010). However, we have never found TcII in the domestics cycle in our study area in previous studies (Diosque et al., 2003; Ragone et al., 2012), nor in the present work, in which we used a sensitive molecular tool. Another interesting observation is the presence of TcIII in the domestic cycle. TcIII is a DTU predominantly found in wild cycles of transmission. Indeed, we have never detected TcIII during a period of about 10 years studying the domestic cycles in this area. However, we have recently isolated this DTU from a dog in the area (Ragone et al., 2012); and it was also reported in the Chaco region of Argentina (infecting dogs and *T. infestans*) in recent years (Cardinal et al., 2008; Enriquez et al., 2013). In the present study we found TcIII in mixed infections with TcI and TcV in dog samples and, for the first time to our knowledge, TcIII was detected infecting humans in this region. Altogether, these results make us wonder whether TcIII could be emerging in the domestic cycle of the Argentinian Chaco. Another possible explanation for the TcIII infection in dogs and humans is the contact of these hosts with infected wild mammals during hunting. There is at least one more possible explanation for the detection of TcIII in a human in our study area: it is possible that TcIII was not previously detected because of low sensitivity of the isolation techniques used in our previous studies. More studies are needed to elucidate if TcIII is emerging in the domestic cycle of the Argentinian Chaco.

We found that 13.5% (18/133) of samples did not hybridize with the used probes. We think that in these samples TcV and TcVI were not present, since these two DTUs show a low genetic diversity and in previous studies using mHVR probes showed that probes are DTU specific for TcV and TcVI. Conversely, TcI show a high genetic diversity in this area of the Chaco region (Diosque et al., 2003; Tomasini et al., 2011; Lauthier et al., 2012). This high genetic diversity within TcI in the study area leads us to suppose that the *T. cruzi* genotypes present in samples that did not hybridized with any of the used probes are probably TcI genotypes different from the used for TcI probe construction. This hypothesis is supported by results of other authors showing that mHVR from certain genotypes of TcI only hybridize with closely related genotypes but not with other genotypes more genetically distant (Brenière et al., 1998). It is worth noting that TcIII also shows a significant genetic diversity (Llewellyn et al., 2009). In fact, we have identified different genotypes of this DTU by using other molecular techniques in the same area (unpublished data). Consequently, we cannot rule out that the unidentified samples may be TcIII genotypes different from the one that we used for construction of the TcIII probe.

Altogether, our results support the idea that in spite of been circulating in the same domestic cycle, there are significant association of TcV with humans and of TcVI with dogs. However, these associations are not exclusive as both, TcV and TcVI, were found infecting humans and dogs in mixed infections. Causes of these associations remain to be elucidated. Finally, the recurrent detection of TcIII in the domestic cycle and its detection infecting humans suggest that changes in the pattern of transmission could be occurring in this area.

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