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Discrete typing units of *Trypanosoma cruzi* identified in rural dogs and cats in the humid Argentinean Chaco

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SUMMARY

The discrete typing units (DTUs) of *Trypanosoma cruzi* that infect domestic dogs and cats have rarely been studied. With this purpose we conducted a cross-sectional xenodiagnostic survey of dog and cat populations residing in two infested rural villages in Pampa del Indio, in the humid Argentine Chaco. Parasites were isolated by culture from 44 dogs and 12 cats with a positive xenodiagnosis. DTUs were identified from parasite culture samples using a strategy based on multiple polymerase-chain reactions. TcVI was identified in 37 of 44 dogs and in 10 of 12 cats, whereas TcV was identified in five dogs and in two cats –a new finding for cats. No mixed infections were detected. The occurrence of two dogs infected with TcIII –classically found in armadillos– suggests a probable link with the local sylvatic transmission cycle involving *Dasypus novemcinctus* armadillos and a potential risk of human infection with TcIII. Our study reinforces the importance of dogs and cats as domestic reservoir hosts and sources of various DTUs infecting humans, and suggests a link between dogs and the sylvatic transmission cycle of TcIII.

Keywords

Chagas disease; *Trypanosoma cruzi*; Discrete Typing Units; dogs; cats; reservoir

INTRODUCTION

Trypanosoma cruzi has a large genetic diversity (Brisse *et al.* 2001) classified into six Discrete Typing Units (DTUs), *T. cruzi* I-VI (Zingales *et al.* 2009). DTUs are distributed differentially among vectors, mammalian hosts and geographic regions. TcIII and TcIV usually occur in sylvatic transmission cycles; TcII, TcV and TcVI occur in domestic cycles, and TcI in both cycles (Yeo *et al.* 2005; Miles *et al.* 2009).

Dogs and cats are major reservoir hosts of *T. cruzi* in domestic transmission cycles throughout the Americas (Mott *et al.* 1978; Gürtler *et al.* 2005, 2007; Crisante *et al.* 2006). Both host species usually display a large fraction of infectious individuals and persistent infectiousness to the vector (Gürtler *et al.* 2007). Dogs are also sensitive sentinels of domestic transmission (Castañera *et al.* 1998; Cardinal *et al.* 2006) and the household

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presence of dogs and cats seropositive for *T. cruzi* increases the risk of parasite transmission to local vectors and humans (Gürtler *et al.* 2005).

Despite the large contribution of both reservoir hosts to the domestic transmission of *T. cruzi*, very few studies have identified the DTUs infecting dogs and cats (Chapman *et al.* 1984; de Luca d'Oro *et al.* 1993; Diosque *et al.* 2003; Cardinal *et al.* 2008; Marcili *et al.* 2009a; Rimoldi *et al.* 2012). In the southern cone countries of South America, the main DTUs identified from humans, domestic dogs and triatomine bugs were TcI, TcII, TcIII, TcV and TcVI (de Luca d'Oro *et al.* 1993; Barnabé *et al.* 2001, 2011; Diosque *et al.* 2003; Cardinal *et al.* 2008; del Puerto *et al.* 2010; Tomasini *et al.* 2011; Yeo *et al.* 2011; Cura *et al.* 2012). In northern Argentina, domestic *Triatoma infestans* and dogs were very frequently infected with TcVI in Santiago del Estero and Chaco provinces (Diosque *et al.* 2003; Cardinal *et al.* 2008).

As part of a multi-site research program on the eco-epidemiology and control of Chagas disease in the Gran Chaco ecoregion, we sought to identify the DTUs infecting domestic dogs and cats to characterize the structure of transmission cycles of *T. cruzi* in a well-defined rural area of the Argentinean Chaco. Parallel efforts in our study area identified the occurrence of TcIII in *Dasypus novemcinctus* armadillos; TcI in *Didelphis albiventris* opossums and peridomestic *Triatoma sordida*; TcV and TcVI in domestic or peridomestic *T. infestans*, and TcVI in peridomestic *T. sordida* (Maffey *et al.* in press; Alvarado-Otegui *et al.* 2012). We found that TcVI was more prevalent in the study dogs and cats followed by TcV; and the occurrence of the typically sylvatic DTU TcIII in two domestic dogs.

MATERIALS AND METHODS

Study area

Field work was conducted in the municipality of Pampa del Indio (26° 2′ 0″ S, 59° 55′ 0″ O), Chaco Province, Argentina, located in the humid (eastern) Chaco, close to the transition to the dry (western) Chaco (Gurevitz *et al.* 2011). The study area included 13 contiguous villages with 323 houses inhabited by two ethnic groups, Creoles and Tobas. Houses were immersed in a landscape consisting of a mosaic of crops and small patches of native dry forest with different degrees of degradation. In most cases house compounds rarely had fences and domestic animals (dog, cat, cattle, goat and sheep) ranged freely in the nearby forest. Most houses were made of mud walls, with metal or thatched roofs, and included peridomestic structures that housed various domestic animals. A cross-sectional demographic and entomologic survey of all study villages revealed that 40% of houses were infested with *T. infestans*; the prevalence of house infestation was 58.8% for Toba households and 43.5% for Creoles' (Gurevitz *et al.* 2011). Prior to community-wide residual spraying with pyrethroid insecticides in December 2007, 27.4% of bugs were infected with *T. cruzi* and bug infection was more frequent in Toba households (42.0%) than in Creoles (20.7%) (Cardinal *et al.* in review).

Study design

A demographic and sero-parasitological survey targeting all dogs and cats residing in seven contiguous villages in August-December 2008 revealed that the overall prevalence of *T. cruzi* infection was 26% in dogs and 29% in cats examined by serological methods and/or xenodiagnosis and the distribution of infected dogs or cats was aggregated at the household level (Cardinal *et al.* in review).

Nested within this larger survey, for the present study we selected two villages (10 de Mayo and Las Chuñas) which had high prevalence of infection with *T. cruzi* in *T. infestans* (61%) before interventions. Then we selected all houses that had at least one *T. cruzi*-infected *T.*

infestans before interventions (38 of 60 inhabited houses) and conducted a xenodiagnostic survey of all dogs and cats. This procedure sought to increase the chances of isolating parasites from a large number of infected individuals. In addition, we included seven dogs and four cats from 6 neighboring communities which had a positive xenodiagnosis. Each head of household was informed on the objectives and relevance of the study; informed oral consent was requested and obtained in all cases. Processing of dogs and cats was conducted according to the protocol approved by the "Dr. Carlos Barclay" Independent Ethical Committee for Clinical Research from Buenos Aires, Argentina (IRB No. 00001678, NIH registered, Protocol N° TW-01-004).

Xenodiagnosis

Dogs were fitted with a muzzle (except newborn pups) and handled with the help of its owners when it did not entail any physical risk, whereas cats were captured by hand or with a net and anesthetized as described elsewhere (Cardinal *et al.* in review). All available dogs and cats were examined by xenodiagnosis using 20 uninfected fourth-instar nymphs of *T. infestans* or 10 nymphs for small pups and kittens (Gürtler *et al.* 2007). All insects were mass-reared and provided by the National Coordination of Vector Control (Cordoba, Argentina). Xenodiagnosis boxes were exposed during 25 minutes on the belly of each individual animal, and a new re-exposure period of 10 minutes followed if most bugs had not blood-fed to repletion. The boxes were held in a cooler to avoid sudden temperature changes during field work, and bugs were kept until examination with no additional blood-feeding.

Faeces from two insects fed on each animal were examined individually at optical microscope (OM) at 400× at 30 days post-exposure, and if negative, the remainder of insects fed on the same individual was analyzed in pools of 4-5 insects each. When the first two bugs or any pool were positive, faeces from of all insects in the positive pool were re-examined individually. Bugs negative at 30 days post-exposure were re-examined individually 30 days later.

Parasite isolation and DTU identification

Parasite culture in biphasic medium and isolation were performed as described elsewhere (Lauricella *et al.* 2005). Briefly, faeces from two OM-positive bugs from each xenodiagnosis-positive animal were separately inoculated into two culture tubes each containing biphasic medium. Parasite growth was monitored weekly during four months until reaching 3×10^5 parasites/ml.

Parasites used for identification of DTUs were obtained from an aliquot of cultures at the time of cryopreservation or when a new sub-culture was initiated after cryopreservation (i.e., when parasite concentration was high). DNA was extracted by boiling parasite pellets as described by Marcet *et al.* (2006). *T. cruzi* DTUs were identified by polymerase-chain reactions (PCR) targeted to three genomic markers: spliced-leader (SL) DNA, 24Sα ribosomal RNA genes and A10 marker using *Taq* DNA polymerase (Invitrogen, USA) as described by Burgos *et al.* (2007) (Figure 1). PCR products were analyzed in 2.5% agarose gels (Invitrogen, USA) for SL-IR I and SL-IR II, in 3% agarose gels for the remainder, and all UV visualization were done after staining with Gel Red (GenBiotech).

Amplification of the SL-IR included the following three independent PCRs: SL IRac (using primers TCac and UTCC) to distinguish TcIII and TcIV (200 bp) from TcI (150 bp), TcII, TcV, and TcVI (157 bp); SL-IR I (using primers TC2 and UTCC) to identify TcI (475 bp); and SL-IR II (using primers TC1-UTCC) to identify TcII, TcV, and TcVI (425 bp) (Figure 1).

Regarding the 24Sα ribosomal RNA genes, a dimorphic region within the D7 domain was amplified by heminested PCR to distinguish TcV (125 or 125 + 140 bp) from TcII and TcVI (140 bp), and TcIII (125 bp) from TcIV (140 bp). The first round PCR was performed using D75 and D76 primers. The heminested round was carried out using 1 μl of the first round PCR in a 30 μl vol. reaction using primers D71 and D76 (Figure 1).

Finally, a heminested PCR targeted to the A10 genomic marker was used to discriminate TcII (580 bp) from TcV and TcVI (525 bp). The first round was carried out using Pr1 and P6 primers whereas the heminested round was carried out using 5 μl of the first round PCR in a 30 μl vol. reaction with primers Pr1 and Pr3 (Figure 1). In summary, 6 PCR assays were necessary to distinguish all UDTs.

Burgos *et al.* (2007) reported that some TcV-infected samples amplified both 125 and 140 bp ribosomal DNA bands in the heminested 24Sα rDNA-PCR (primers D71 and D76) (Figure 2B). When this pattern appeared, it was not possible to exclude an infection with TcVI (i.e., differentiate single infections with TcV from mixed infections with both TcV and TcVI). In our samples, all parasite stocks infected with TcV amplified both bands. Consequently, we also considered the results from the first round of the 24Sα rDNA-PCR (primers D75 and D76) to distinguish infections with TcV only (showing a 275 bp band only) from those with TcV and TcVI (275 and 290 bp bands) (Figure 2A). *T. cruzi* reference stocks used as controls were TcI (X-10 and CA-1 K98); TcII (Tu 18); TcIII (M5631); TcIV (Can III); TcV (PAH 265); and TVI (Cl-Brener).

RESULTS

A total of 39 of 100 dogs and 8 of 29 cats examined with xenodiagnosis were positive at the two study villages. Parasites were successfully isolated from 37 of 39 dogs and from all 8 cats positive by xenodiagnosis, as well as from seven dogs and four cats residing in neighbouring communities. DTUs were identified in 56 culture-derived DNA samples (Table 1). TcVI was found in 37 of 44 dogs and in 10 of 12 cats. TcV was identified in five dogs and two cats, whereas TcIII was found only in two dogs. No mixed infections were detected.

At the household level, TcVI predominated in 26 of 30 houses with DTUs identified in dogs or cats (Table 2). TcVI was found alone in 22 houses and co-circulated with TcV in four houses. TcIII and TcV occurred in two houses each, in none of which TcVI was identified.

DISCUSSION

Our study documents the predominance of TcVI and the occurrence of TcV in xenodiagnosis-positive dogs and cats in a rural endemic area in the humid Argentinean Chaco, in the largest survey of DTUs infecting dogs and cats conducted so far. We also provide the first report of two cats infected with TcV.

Although previously reported in the Gran Chaco, the occurrence of TcV and TcVI has rarely been documented in non-humans hosts (Miles *et al.* 2009; Zingales *et al.* 2012). The predominance of TcVI in parasite stocks from dogs and cats is consistent with the predominance of TcVI in local *T. infestans*, especially in bugs collected from peridomestic kitchens and storerooms where they usually rested (Maffey *et al.* in press). The role of dogs and cats as sources of *T. cruzi* was also evidenced by the finding of a strong association between dog and bug infection at the household level (Gürtler *et al.* 2005). Given the relevance of peridomestic foci in the process of house reinfestation with *T. infestans* after residual spraying with insecticides (Cecere *et al.* 2004; Gurevitz *et al.* in press), dogs and

cats may be the first sources of infection for the bugs that re-invade the house compound, and their DTUs may be involved in renewed parasite transmission after interventions.

Most houses had dogs and cats infected with TcVI only, despite the study identified DTUs from up to 2-5 animals at the same house. The predominance of TcVI in domestic dogs also occurred in other areas in the dry Argentinean and Paraguayan Chaco (Chapman *et al.* 1984; Diosque *et al.* 2003; Cardinal *et al.* 2008), suggesting an association between TcVI and peripheral blood infection of dogs and cats. We cannot exclude the occurrence of undetected mixed infections produced by histotropism within the mammalian host (Vago *et al.* 2000; Burgos *et al.* 2010) or by selection of genotypes during parasite isolation and culture, as was revealed by parasite cloning in agar plates (Yeo *et al.* 2007).

DTUs typically associated with the sylvatic transmission cycle have been identified in dogs with very low frequencies. In the Argentinean dry Chaco, TcIII was identified in 3 of 31 infected dogs from Santiago del Estero Province (Cardinal *et al.* 2008) whereas no TcIII infections were recorded in 16 infected dogs from Chaco and in two dogs from Santiago del Estero (de Luca d'Oro *et al.* 1993; Diosque *et al.* 2003). In the Paraguayan Chaco, TcIII was identified in 3 of 7 infected dogs (Barnabé *et al.* 2001, based on isoenzyme patterns in Chapman *et al.* 1984). Cats have been found to be infected only with TcVI prior to our current study (Cardinal *et al.* 2008). In Pampa del Indio, parallel surveys identified TcIII in armadillos *Dasypus novemcinctus* and TcI in *Didelphis albiventris* and in adult *Triatoma sordida* collected in peridomestic structures (Alvarado-Otegui *et al.* 2012; Maffey *et al.* in press). In spite of the local occurrence of these DTUs, we only detected TcIII in two of 44 infected dogs. Regardless of the exact mode of transmission underlying such infections (i.e., oral transmission from hunting and eating armadillos, vector-mediated transmission or vertical transmission), these findings demonstrate the occurrence of a typically sylvatic DTU in domestic dogs and a potential risk of human infection with TcIII because *T. infestans* and other domestic bug species frequently blood-feed on dogs (Gürtler *et al.* 2007).

This study documents the finding of both domestic and sylvatic DTUs infecting domestic dogs. Previous studies in South America have rarely found TcIII in the domestic environment, where it is more prevalent in dogs than in humans, whereas TcIII occurs more frequently in domestic hosts than TcV or TcVI in sylvatic mammals (Miles *et al.* 2009; Zingales *et al.* 2012). These results imply a great versatility of the dog reservoir in harbouring a broad genetic diversity of *T. cruzi*, and the need for more research on the role of dogs in *T. cruzi* transmission across the Americas up to the US.

With the aim of fully describing the genetic diversity of *T. cruzi*, high-resolution methods have recently been applied to investigate parasite populations at the infra-DTU level, such as microsatellites (Llewellyn *et al.* 2009) or multilocus sequence typing (Yeo *et al.* 2011). Our study may be considered a first step toward the understanding of the structure of *T. cruzi* transmission cycles through the distribution of DTUs in dogs and cats in a well-defined area. Those novel tools are needed to probe the local genetic structure of *T. cruzi* with greater detail, and examine the putative sylvatic origin of dog infections with TcIII and TcI.

Previous studies conducted in Argentina recorded TcI, TcV and TcVI in human patients affected by Chagas disease (Schijman *et al.* 2004; Burgos *et al.* 2007, 2010; Cura *et al.* 2012). All of these human-infecting DTUs were found in domestic dogs and cats from Chaco and Santiago del Estero Provinces further evidencing their relevance as reservoir hosts of *T. cruzi* in the domestic transmission.

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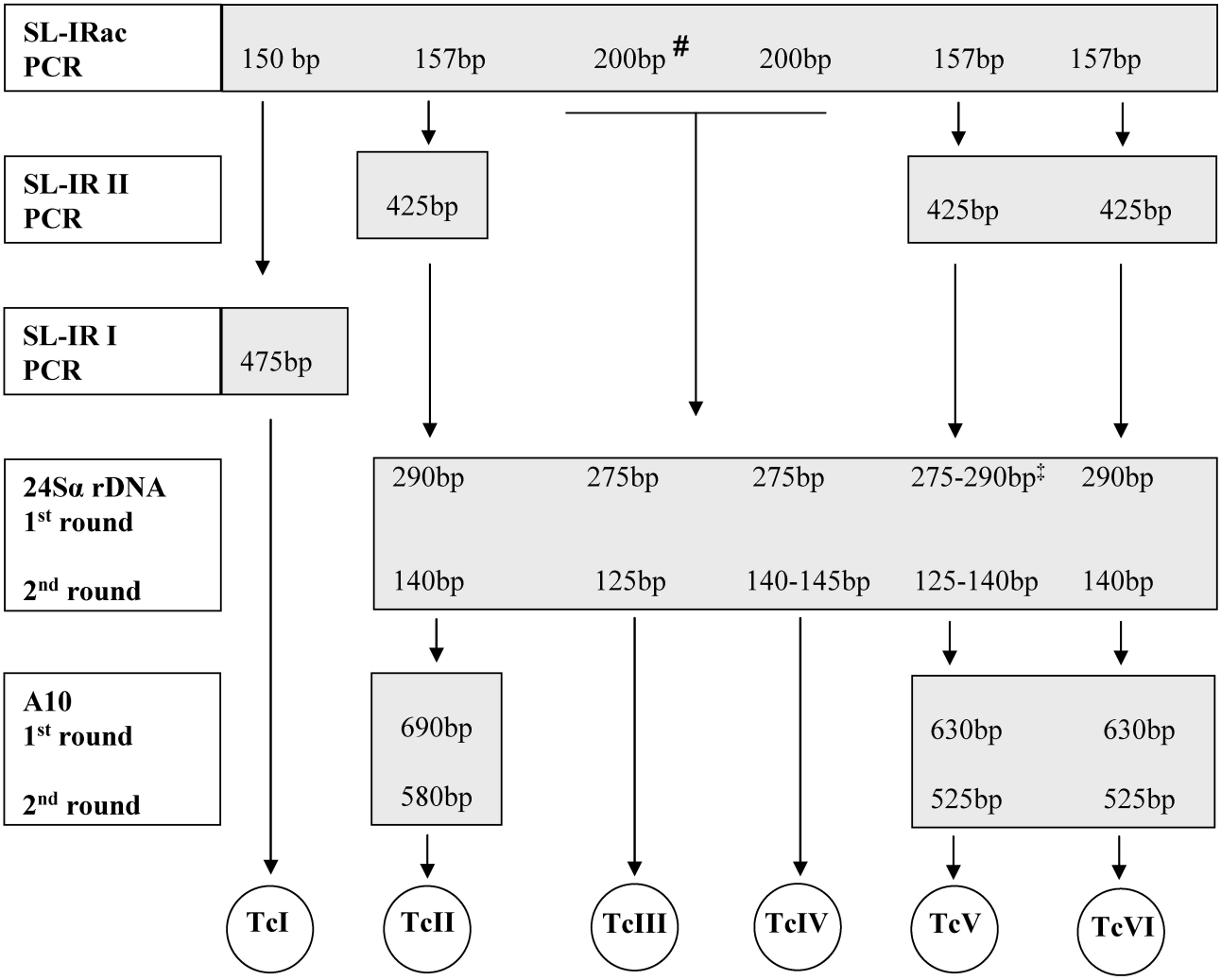

An additional 150 bp band may occasionally appear.
‡ Only one (275 or 290 bp) or both bands (275+290 bp) can be amplified in the 24Sα-rDNA first round PCR.

Figure 1.
Decision key for discriminating *T. cruzi* DTUs following Burgos et al (2007).

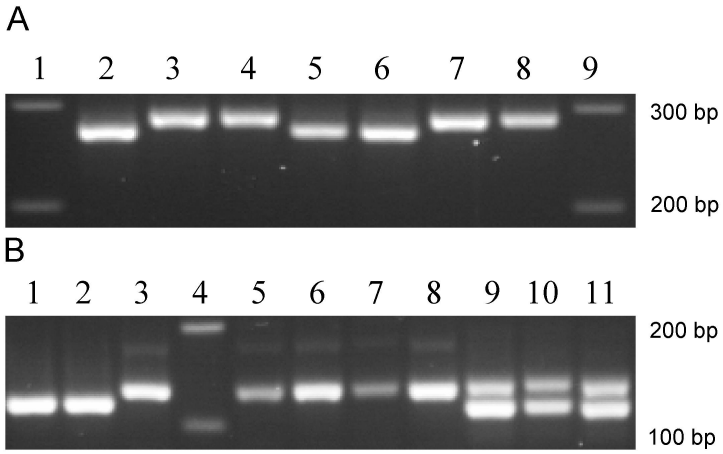

Figure 2.

Example of 24Sα-rDNA PCR product size polymorphism A) First round PCR, primers D75 and D76 Lane (1) 100 bp ladder; lane (2) reference stock PAH 265 (TcV); lane (3) reference stock CL-Brener (TcVI); lane (4) reference stock Tu 18 (TcII); lane (5) sample of dog with identified DTU (TcV); lane (6) sample of cat with identified DTU (TcV); lane (7) sample of dog with identified DTU (TcVI); lane (8) sample of cat with identified DTU (TcVI); lane (9) 100 bp ladder. B) Heminested PCR, primers D71 and D76 Lane (1) sample of dog with identified DTU (TcIII); lane (2) reference stock M5631 (TcIII); lane (3) reference stock Can III (TcIV); lane (4) 100 bp ladder; lane (5) sample of dog with TcVI identified; lane (6) reference stock Tu 18 (TcII); lane (7) sample of cat with identified TcVI; lane (8) reference stock CL-Brener (TcVI); lane (9) reference stock PAH 265 (TcV); lane (10) sample of dog with TcV identified; lane (11) sample of cat with TcV identified.

Table 1

Distribution of Discrete Typing Units (DTUs) of *T. cruzi* in isolates from dogs and cats, Pampa del Indio, Chaco, 2008

	No. of individuals with DTU identified			
Host	**TcIII**	**TcV**	**TcVI**	**Total**
Dog	2	5	37	44
Cat	0	2	10	12
Total	2	7	47	56

Table 2

Distribution of Discrete Typing Units (DTUs) in house compounds where *T. cruzi* was isolated from dogs or cats

	Number of houses			
DTU	**Only in dogs**	**Only in cats**	**Both hosts**	**Total**
Only TcVI	14	5	3	22
Only TcIII	2	0	0	2
Only TcV	1	0	1	2
TcV and TcVI[†]	2	0	2[‡]	4
Total	19	5	6	30

[†]Single infections with TcV and TcVI were identified in different hosts residing at the same house compound.

[‡]Includes one house where TcV was identified in one cat and TcVI in both a cat and a dog, and another house with TcV in a dog and TcVI in a cat.