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Trypanosoma cruzi infection in *Triatoma sordida* before and after community-wide residual insecticide spraying in the Argentinean Chaco

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ABSTRACT

Triatoma sordida is a secondary vector of *Trypanosoma cruzi* in the Gran Chaco and Cerrado eco-regions where it frequently infests peridomestic and domestic habitats. In a well-defined area of the humid Argentine Chaco, very few *T. sordida* were found infected when examined by optical microscopic examination (OM). In order to further assess the role of *T. sordida* and the relative magnitude of subpatent bug infections, we examined the insects for *T. cruzi* infection, parasite Discrete Typing Units (DTUs) and bloodmeal sources using various molecular techniques. Among 205 bugs with a negative or no OM-based diagnosis, the prevalence of infection determined by kDNA-PCR was nearly the same in bugs captured before (6.3%) and 4 months after insecticide spraying (6.4%). On average, these estimates were six-fold higher than the prevalence of infection based on OM (1.1%). Only TcI was identified, a DTU typically associated with opossums and rodents. Chickens and turkeys were the only bloodmeal sources identified in the infected specimens and the main local hosts at the bugs' capture sites. As birds are refractory to *T. cruzi* infection, further studies are needed to identify the infectious bloodmeal hosts. The persistent finding of infected *T. sordida* after community-wide insecticide spraying highlights the need of sustained vector surveillance to effectively prevent *T. cruzi* transmission in the domestic and peridomestic habitats.

RELEVANT KEY WORDS

Triatoma sordida, *Trypanosoma cruzi*, molecular diagnosis, DTU, bloodmeal identification, vector control

1. INTRODUCTION

The role of various species of Triatominae as secondary vectors of *Trypanosoma cruzi*, the etiological agent of Chagas disease, remains controversial (Guhl et al., 2009). This controversy probably reflects the richness of species involved, their broad geographic range, the dynamic nature of the domestic invasion process, and the limited research efforts invested in sylvatic species of Triatominae. *Triatoma sordida* (Stål, 1859) is a secondary vector of *Trypanosoma cruzi*, exhibiting different degrees of domiciliation and colonization especially in areas where the main vector *Triatoma infestans* was suppressed (Forattini et al., 1983; Noireau et al., 1997). *Triatoma sordida* is widely distributed across northern Argentina, eastern Bolivia, Paraguay and southeastern Brazil (Carcavallo et al., 1999). It is frequently found in tree holes, under bark, palm trees, bromeliads, small mammals' burrows, and bird nests, and usually colonizes peridomestic chicken coops or nests (Diotaiuti et al., 1993). The prevalence of *T. cruzi* infection in *T. sordida* was usually less than 2% (Oscherov et al., 2003), and given that this species shows little anthropophily, it was considered of low epidemiological significance across most of its geographic range (Silveira 2002) except in Bolivia where it reached 21% (Noireau et al., 1997; Brenière et al., 1998).

Domestic and sylvatic transmission cycles have been characterized according to host, vector species and habitats, and may be separated or overlap to various degrees. The six parasite genotypes identified (TcI-TcVI), called Discrete Typing Units (DTUs), usually exhibit different distributions between transmissions cycles (Zingales et al., 2012). In domestic transmission cycles of the Argentine Chaco, TcV and TcVI were the predominant DTUs identified in *T. infestans*, domestic dogs, cats and humans (Diosque et al., 2003;

Cardinal et al., 2008; Enriquez et al., 2013; Maffey et al., 2012). The main sylvatic reservoir hosts identified were *Didelphis albiventris* opossums infected with TcI, and armadillos and *Conepatus chinga* skunks infected with TcIII (Diosque et al., 2003; Orozco et al., 2013). Several rodent species exhibited subpatent infections only revealed by kDNA-PCR (Orozco et al., 2014). The putative sylvatic vectors of *T. cruzi* have not been conclusively identified yet in the Gran Chaco eco-region. Particularly, in Pampa del Indio (Argentine Chaco), *T. sordida* and *Panstrongylus geniculatus* captured in sylvatic habitats were not found to be infected (Alvarado-Otegui et al., 2012). The predominant DTUs identified in the very few (n = 9) OM-positive peridomestic *T. sordida* examined were TcVI (56%) and TcI (33%), leaving unclear the role of this species in the domestic transmission cycle (Maffey et al., 2012). These findings prompted us to further examine *T. sordida* bugs for *T. cruzi* infection, DTUs and bloodmeal sources using molecular methods to elucidate its role as a secondary vector across a diversity of rural villages, seasons and bug-control contexts. We included a sizable number of *T. sordida* specimens captured in peridomestic habitats and all the *T. sordida* captured in domiciles before and after full-coverage house spraying with insecticides. Our hypotheses were that *T. sordida* harbored subpatent infections with *T. cruzi*, and that *D. albiventris* opossums were the bloodmeal source of TcI-infected bugs.

2. MATERIALS AND METHODS

2.1 Study area

The present study was carried out in a rural section (450 km²) of Pampa del Indio Municipality (25° 55'S 56°58'W), Province of Chaco, Argentina, comprising 327 inhabited households belonging to 13 communities which have already been described elsewhere (Gurevitz et al., 2011).

2.2 Entomological surveys

A baseline (BL) survey was conducted in all households in the study area to assess triatomine infestation levels by timed manual searches (TMS) using a flushing-out agent in September-December 2007 (Gurevitz et al., 2011). Immediately after the BL survey, all households were sprayed with residual pyrethroid insecticide and house reinfestation was re-assessed at 4 months post-spraying (4 MPS). In several houses bugs were also collected by other methods (manually after timed-manual searches, i.e. post-TMS; during insecticide application, and by householders between insecticide spraying and 4 MPS). These additional bug collections were used to estimate the prevalence of house infestation at both surveys.

All collected triatomines were identified to species following Lent & Wygodzinsky (1979), stage and sex as described elsewhere (Canale et al., 2000). The taxonomy of the *T. sordida* complex has not been completely resolved (Calderón-Fernández and Juárez 2013) and it is frequently difficult to distinguish the various member species based on external morphology only. Therefore, the study specimens may be considered as *T. sordida* 'sensu lato'.

Microscopic examination for *T. cruzi* infection (OM) was restricted to live third-instar nymphs and later stages prioritizing *T. infestans* over *T. sordida* specimens. Bugs were examined at 400× within 20 days of collection, and preserved at -20 °C.

2.3 Study design

Our sampling design aimed at analyzing *T. sordida* from all infested households. Adult bugs and fourth- or fifth-instar nymphs with a negative optical microscopy (OM)–diagnosis or not examined for infection were selected for kDNA-PCR. Five or less specimens of *T. sordida* were collected in most households infested by this species: 73% at BL and 72% at 4 MPS, and all of these bugs were examined for infection. In households with larger infestations, 5 bugs were randomly selected among adults and late nymphal stages. Households not included in the study sample usually harbored early nymphal stages or only OM-positive bugs (reported in Maffey et al., 2012). Each selected insect was dissected, and the rectal ampoule, abdomen and rest of the insect separated in labeled microtubes as described in Maffey et al. (2012).

2.4 PCR-based diagnosis of *T. cruzi* infection

DNA extraction from rectal ampoules' contents was performed using a commercial reagent (DNAzol, Life Technologies) to reduce the presence of inhibitors. Infection by *T. cruzi* was determined using a hot-start PCR targeting a 330 bp amplicon of the kinetoplast minicircle (kDNA-PCR) following standardized protocols (Maffey et al., 2012). In kDNA-PCR positive insects, another hot-start PCR was performed to confirm *T. cruzi* infection by targeting a *T. cruzi* satellite sequence (SAT-DNA-PCR) (Orozco et al., 2013). PCR

products were analyzed in 3% agarose gels (Invitrogen, USA) and UV visualization after staining with Gel Red (GenBiotech).

2.5 Identification of parasite DTUs

DTUs were identified by PCR strategies directed to the spliced-leader sequence (SL, or "mini-exon"), alpha 24s rDNA, and A10 genomic marker following the protocol described by Burgos et al. (2007). Hemi-nested SL-PCRs using primers TCC-TC1 or TCC-TC2 were also performed to increase sensitivity as described in Enriquez et al. (2014).

2.6 Bloodmeal identification

Given that most infected *T. sordida* had scarce bloodmeal contents, the abdomens were further cut into small pieces and mixed thoroughly with PBS to separate the stomach contents from abdomen walls. DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kit, QIAGEN Sciences, Maryland, USA) and used as a template for PCR targeting cytochrome b gene (CytB), following the protocol specified in Lee et al. (2002). To identify the bloodmeal source, amplified products were sent to an external service (Macrogen Inc., Seoul, Korea) for automatic sequencing. Sequences were manually aligned using MEGA 5.1 software (Tamura et al., 2011). A consensus of forward and reverse sequences was created and a BLAST search was performed in GenBank database to compare sequence identity. Sequences have been submitted to GenBank (KP231499, KP231500).

2.7 Data analysis

Proportions were compared using Fisher's exact test. Wilson 95% confidence intervals (CI_{95}) were calculated for proportions. Baseline and 4 MPS entomological data were pooled together in order to perform spatial analyses. *Triatoma sordida*-infested households and houses with infected bugs were incorporated in a GIS database of all houses of the study area using ArcGis 9.1. Infected *T. sordida* reported in Maffey et al. (2012) were also included. To test whether *T. sordida*-infested households and *T. sordida*-infested households were randomly distributed, we first performed a global spatial analysis (K-function and weighted K-function, respectively). Local spatial analysis was performed using the Getis $G_i^*(d)$ statistic to explore whether *T. sordida*-infested households were aggregated in space (Getis and Ord 1996). Spatial analyses were performed using Point Pattern Analysis software (San Diego State University, San Diego, CA; Chen and Getis 1998).

3. RESULTS

3.1 *T. sordida* infestation

A total of 518 and 281 *T. sordida* was collected using all methods at BL and 4 MPS, respectively. The prevalence of house infestation by *T. sordida* decreased significantly from 21.1% at BL to 13.5% at 4 MPS (Fisher's exact test, $p = 0.01$). The median bug abundance per infested site was 2 (first-third quartiles: Q1-Q3 = 1-4) at BL and 1 (Q1-Q3 = 1-4) at 4 MPS. Most bugs were collected in peridomiciles. The most frequent sites of capture were ecotopes associated with chickens (63.5% at BL and 67.4% at 4 MPS). Only 2 bugs were collected in domiciles in each survey, and they occurred in different households.

3.2 *Trypanosoma cruzi* infection

A total of 148 (46.0%) of 322 *T. sordida* captured by TMS at BL and 128 (67.7%) of 189 captured at 4 MPS were examined for *T. cruzi* infection by OM. The prevalence of bug infection determined by OM was 2.0% at BL and no infected bug was found at 4 MPS (Table 1). For kDNA-PCR diagnosis, we selected 127 *T. sordida* at BL and 78 bugs at 4 MPS, representing 39.4% and 41.3% of the total collected by TMS, respectively. Overall, 83.3% ($n = 54$) of the infested households at BL and 97.4% ($n = 39$) of those infested at 4 MPS were included in the study sample (Table 1). Using kDNA-PCR, the prevalence of infection was three-fold higher than by OM and marginally significant at BL (6.3%, 8 cases) (one-sided Fisher's exact test, $p = 0.07$), and significantly higher at 4 MPS (6.4%, 5 cases) (one-sided Fisher's exact test, $p = 0.007$). No significant differences were found between bug infection rates at BL and 4 MPS for each detection method (Fisher's exact tests, $p > 0.25$ for both) (Table 1). We confirmed *T. cruzi* infections by SAT-DNA-PCR in 7 out of the 13 individuals that were kDNA-PCR positive (Table 2). Among the 205 bugs

selected for PCR analysis, 111 had previously been OM-negative, and therefore the false-negative rate was 2.7%.

The infected *T. sordida* identified by PCR were mainly adults ($n = 10$), followed by 2 fifth instars and 1 fourth-instar nymph. All of the infected bugs were captured in ecotopes associated with chickens and in different households, except for a female captured in a corn storage shed and 2 bugs collected in a chicken coop from the same household (Table 2).

3.3 DTU identification

TcI was the only DTU detected in both surveys (Table 2). Four out of 13 infected bugs were not reactive to any of the PCRs used for DTU identification including hemi-nested SL-PCRs.

Considering all the 18 infected *T. sordida* with successful DTU identification (including those in Maffey et al., 2012), a significant association between DTU and bug capture ecotope was found. TcI was significantly associated with ecotopes used by chickens, and TcVI or TcV with other ecotopes (domiciles, kitchen and corral) (Fisher's exact test, $p = 0.004$) (Figure 1).

3.4 Spatial analysis of *T. cruzi* infection

Households with infected *T. sordida* were not aggregated at a global scale. However, local aggregation was found and a hotspot detected within a radius of 4.2 km at Los Ciervos village (Figure 2).

3.5 Bloodmeal sources

Amplification of vertebrate cytochrome b was achieved in all of the infected bugs, but successful bloodmeal identification was possible only in 9 individuals. Sequence analysis showed high similarity (78-100% identity,) with *Gallus gallus* (chicken) for 8 bugs (**KP231500**), and 100% identity with *Meleagris gallopavo* (wild turkey) for the remainder (**KP231499**) (Table 2). All bugs but one were captured in ecotopes associated with chickens (Table 2).

4. DISCUSSION

Our study shows a six-fold increase of *T. cruzi* prevalence in *T. sordida* as determined by means of highly sensitive PCR tests relative to standard OM-based examination. The false-negative rate was very low, as expected from the very low prevalence of infection in this vector species. PCR-based analysis is to be preferred relative to OM for the diagnosis of *T. cruzi* infection in *T. sordida*. This species usually feeds on poultry blood, characterized by a lower nutritional value than mammalian blood (Lehane, 1991), leading to a poorer nutritional status and lower intensity of infection (Schaub, 2009), which may fail to be detected using OM examination.

No change was observed in the prevalence of infection of *T. sordida* over time after a full-coverage insecticide spraying that strongly reduced the prevalence of house infestation and abundance of *T. infestans* (Gurevitz et al., 2013) and its prevalence of *T. cruzi* infection (Cardinal et al., 2014, unpublished data). These patterns are similar to those described for *T. sordida* in Brazil (Rocha-Silva et al., 1969; Pires et al., 1999).

Although we found no global aggregation of infection in *T. sordida*, significant local aggregation and a hotspot near one of the villages were detected. Interestingly, this pattern is partially coincident with a local hotspot of sylvatic mammal infections including marsupials, armadillos and rodents, which were infected with TcI or TcIII (Orozco et al., 2013, 2014). *Triatoma sordida* may have acquired the infections from some of these sylvatic mammals at the hotspot, as suggested by DTU identifications. The predominant DTU in *T. sordida* was TcI, which is typically associated with *Didelphis* opossums across the Americas (Yeo et al., 2005) including our study area (Orozco et al., 2013), and with sigmodontine and murine rodent infections (reviewed in Orozco et al., 2014). The

predominance of TcI differs from our previous findings in which nearly half (56%) of the OM-positive *T. sordida* had TcVI and only 33% had TcI (Maffey et al., 2012). This discrepancy between studies could be explained by the different sources (ecotopes) of the bugs examined for infection, and the significant association between DTU and bug capture ecotope. *T. sordida* bugs infected with TcV or TcVI were associated with domiciles, storerooms and kitchens, where *T. infestans* made frequent contact with humans, dogs and cats (Gürtler et al., 2014), whereas *T. sordida* infected with TcI were captured in ecotopes associated with chickens.

Bloodmeal identifications were expected to shed light on the host species that originated *T. sordida* infections. However, we only identified blood meals on chickens and other poultry which are refractory to *T. cruzi* infection. Therefore, the infectious blood meals most likely occurred earlier in the rather long life cycle of *T. sordida* spanning approximately one year. The predominance of TcI among *T. sordida* and lack of detection of a potentially infectious blood meal have various possible explanations. First, *T. sordida* may acquire the infection during early nymphal stages from infected opossums or rodents near the houses, and then invade peridomestic structures to blood-feed on chickens. Second, the infections may have been acquired from infected opossums or synanthropic rodents (attracted by food sources or grain) at or nearby the peridomestic habitats where the bugs were collected (Pires et al., 1999). Consistent with this hypothesis, TcI was isolated from *Rattus rattus* in a rural village in Chaco province (Tomasini et al., 2011). Third, domestic dogs and cats are highly infectious hosts and may have served as sources of infection, although a very small fraction of them were infected with TcI in the study area (Enriquez et al., 2014). Unlike elsewhere in South America (Guhl & Ramirez 2013) and in Argentina (Montamat et al., 1996), at present humans apparently rarely constitute a source

of TcI infections since all recently described human TcI infections have been recorded from immunosuppressed patients or heart explant samples (Cura et al., 2012). Assessment of infra-DTU diversity (Llewellyn et al., 2009) among local TcI-infected vectors and hosts may help to identify the origin of these infections. Large amounts of parasite DNA and in vitro culture are required for these purposes.

Our study has both limitations and strengths. Major strengths include the use of highly sensitive molecular techniques to identify bloodmeal sources and infection status, and the intense sampling coverage of houses across a large study area. Our main limitation is due to the small number of infected samples despite considerable efforts to increase the detectability of bug infections with *T. cruzi*. Not all of the kDNA-PCR positive samples could be confirmed by SAT-DNA-PCR, which is expected from the fewer copies of satellite DNA in TcI (Duffy et al., 2009). Because TcI infections predominated in our samples, we consider the kDNA-positive, SAT-negative cases as *T. cruzi* infections. Secondly, the time lag elapsed between bug captures and microscopic examination may have further contributed to a decrease in the intensity of infection and reduced its detectability.

Maffey et al. (2012) suggested *T. sordida* may have a role as a "bridge" vector of *T. cruzi* between domestic and sylvatic transmission cycles. Our results implicate *T. sordida* in the transmission of *T. cruzi* in domestic or peridomestic habitats. The pioneering studies performed by Forattini et al. (1971) assessed the invasive capacity of domestic habitats displayed by *T. sordida* which was subsequently verified by others (Falavigna-Guilherme et al., 2004; Rojas de Arias et al., 2012). Given the association between human infection with *T. cruzi* and domestic populations of *T. sordida* in some sections of Bolivia where *T.*

infestans was not present (Noireau et al., 1997), sustained vector surveillance is needed to monitor the potential role of *T. sordida* as a domestic or peridomestic vector of *T. cruzi*.

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Table 1: Prevalence of *T. cruzi* infection in *T. sordida* at baseline and 4 MPS as determined by kDNA-PCR and optic microscopy (OM).

Survey	OM		kDNA-PCR	
	Percentage of coverage of infested households (N)	Percentage of infected bugs (CI ₉₅)	Percentage of coverage of infested households (N)	Percentage of infected bugs (CI ₉₅)
BL	37.0 (54)	2.0 (0.7-5.8)	83.3 (54)	6.3 (3.2-11.9)
4 MPS	100 (39)	0.0 (0.0-2.9) ^a	97.4 (39)	6.4 (2.8-14.1) ^b
Total	68.7 (83)	1.1 (0.4-3.1) ^a	88.0 (83)	6.3 (3.7-10.5) ^b

N is the number of infested houses; CI₉₅ is the 95% confidence interval.

^{a,b} Different letters indicate significant differences by Fisher's exact tests, $p \leq 0.007$.

Table 2: Origin of the 13 *T. cruzi*- positive *T. sordida* as determined by PCR and additional molecular characterizations for bloodmeal source and parasite DTU, Pampa del Indio, Chaco, 2007-2008.

Survey	Household ID	Collection site	Stage	OM result	SAT-DNA	DTU	Blood meal
BL	LUG 19	Chicken tree	IV	NA	Negative	TcI	Turkey
	LUG 26	Chicken coop	Male	NA	Positive	TcI	Chicken
	LUG 26	Chicken coop	Male	NA	Positive	TcI	Chicken
	CV 2	Chicken coop	V	NA	Positive	TcI	Chicken
	CV 3	Corn storage shed	Female	NA	Negative	TcI	Chicken
	SV 23	Chicken coop	Male	NA	Negative	TcI	Chicken
	3L 9	Chicken coop	Male	NA	Positive	TcI	Chicken
	BV 10	Chicken nest	Female	Negative	Negative	TcI	Chicken
4 MPS	LUG 1	Chicken nest	Female	NA	Negative	NI	NI
	LUG 47	Chicken nest	Male	NA	Positive	NI	NI
	CV 6	Chicken nest	V	Negative	Positive	TcI	NI
	SV 20	Chicken nest	Male	NA	Negative	NI	NI
	RI 19	Chicken tree	Male	Negative	Positive	NI	Chicken

BL: Baseline; 4MPS: 4 month post-spraying; NA: not analyzed; NI: not identified.

Figure 1: Distribution of *T. cruzi* DTUs in *T. sordida* by bug capture ecotope. Figure includes OM-infected *T. sordida* reported by Maffey et al. (2012). ‘Other ecotopes’ includes domicile, kitchen, corral and corn storage shed.

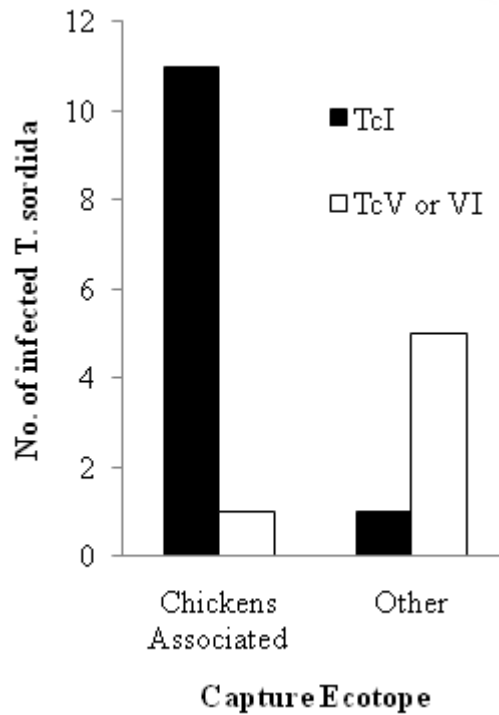


Figure 2: Map of the study area in Pampa del Indio showing houses harboring *T. sordida* (light grey dots), *T. cruzi*-infected bugs (dark grey dots), village Los Ciervos (dotted line) and the hot spot area (shaded with grey). Figure excludes houses not infested with *T. sordida*. See Maffey et al. (2012) for further details.

