Hosts and vectors of *Trypanosoma cruzi* discrete typing units in the Chagas disease endemic region of the Paraguayan Chaco

NIDIA ACOSTA¹, ELSA LÓPEZ¹, MICHAEL D. LEWIS², MARTIN S. LLEWELLYN², ANA GÓMEZ³, FABIOLA ROMÁN³, MICHAEL A. MILES² and MATTHEW YEO²*  

¹ Departamento de Medicina Tropical, Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción – UNA, San Lorenzo CP 2160, Paraguay  
² Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK  
³ Centro para el Desarrollo de la Investigación Científica (CEDIC)/Diaz Gill Medicina Laboratorial/Fundación Moisés Bertoni, Asunción, Paraguay  

(Received 30 October 2016; revised 16 December 2016; accepted 19 December 2016; first published online 9 February 2017)

**SUMMARY**

Active *Trypanosoma cruzi* transmission persists in the Gran Chaco region, which is considered hyperendemic for Chagas disease. Understanding domestic and sylvatic transmission cycles and therefore the relationship between vectors and mammalian hosts is crucial to designing and implementing improved effective control strategies. Here we describe the species of triatomine vectors and the sylvatic mammal reservoirs of *T. cruzi*, in different localities of the Paraguayan and Bolivian Chaco. We identify the *T. cruzi* genotypes discrete typing units (DTUs) and provide a map of their geographical distribution. A total of 1044 triatomines and 138 sylvatic mammals were captured. Five per cent of the triatomines were microscopically positive for *T. cruzi* (55 *Triatoma infestans* from Paraguay and one sylvatic *Triatoma guasayana* from Bolivia) and 17 animals (12·3%) comprising eight of 28 (28·5%) *Dasypus novemcinctus*, four of 27 (14·8%) *Euphractus sexcinctus*, three of 64 (4·7%) *Chaetophractus spp.* and two of 14 (14·3%) *Didelphis albiventris*. The most common DTU infecting domestic triatomine bugs was TcV (64%), followed by TcVI (28%), TcI (6·5%) and TcIII (5·1%). TcIII was overwhelmingly associated with armadillo species. We confirm the primary role of *T. infestans* in domestic transmission, armadillo species as the principal sylvatic hosts of TcIII, and consider the potential risk of TcIII as an agent of Chagas disease in the Chaco.

Key words: *Trypanosoma cruzi*, Paraguayan Chaco, triatomine vectors, armadillos, discrete typing units.

**INTRODUCTION**

*Trypanosoma cruzi* is the causative agent of Chagas disease, a neglected human protozoan disease that is estimated to affect approximately six million people, spanning 21 endemic Latin American countries, with 60–80 million at risk of infection (WHO, 2015). *Trypanosoma cruzi* is genetically heterogenous, infecting a large number of mammal species and transmitted by haematophagous triatomine insect vectors. Nomenclature is historically complicated, but *T. cruzi* is currently subdivided into six subspecific groups, referred to as genetic lineages or discrete typing units (DTUs) and designated TcI to TcVI (Zingales et al. 2012). A cohort of geographically disparate bat trypanosomes, provisionally designated as TcBat, has been shown to share phylogenetically close affiliations with TcI (Marcili et al. 2009a), although more detailed sampling is required to confirm this as a formal taxonomic group. In a recent review, analysing more than 400 sequences with two mitochondrial (*CytB* and *COII*) and one nuclear gene (*Gpi*), authors propose three significant reliable mitochondrial clades, named mtTcI, mtTcII and mtTcIII, instead of seven (Barnabé et al. 2016). Phyloepidemiology and host vector associations of *T. cruzi* are complex, but have been partially resolved (Yeo et al. 2005; Miles et al. 2009; Messenger et al. 2015; Brenière et al. 2016). TcI is widespread through the Americas. This is the major DTU found infecting *Didelphis* opossums in nature, which is believed to be its most ancestral host. TcI was reported predominating in domestic transmission cycles in northern countries of South America (for example, Colombia and Venezuela) and in Central America (Miles et al. 2009). TcIII and TcIV primarily circulate in sylvatic transmission cycles, the former especially associated with armadillos (Yeo et al. 2005) and the latter with a variety of sylvatic mammal species (Miles et al. 2009). TcIV is a secondary cause of Chagas disease in Venezuela. TcIII is rarely reported from domestic transmission cycles. In contrast, TcII, TcV and TVI predominate in domestic transmission cycles in...
Southern Cone countries of South America (Miles et al. 2009; Messenger et al. 2015; Brenière et al. 2016). Remarkably, TcV and TcVI are known to be natural hybrids derived from genetic exchange between TcII and TcIII in recent evolutionary history and are at present strongly associated with domestic transmission cycles (Zingales et al. 2012; Brenière et al. 2016).

Trypanosoma cruzi infection is considered primarily a zoonosis and as such eradication is not possible. Effective control of Chagas disease is achieved by interrupting vectorial transmission, primarily through residual insecticide-spraying to reduce domestic infestation and also by serological surveillance and interruption of transmission by blood transfusion, organ donation and congenitally (WHO, 2015).

There has been remarkable progress in controlling Chagas in some regions of the Americas. However, the Gran Chaco region, which includes territories of Argentina, Bolivia and Paraguay, is currently considered one of the most difficult regions for effective control and remains highly endemic (Hotz, 2014a). The land area is vast and arid, with a low population density consisting of small widely dispersed communities of low socioeconomic status (Gürtler, 2009). More than 20 ethnic groups live in marginalized conditions with minimal access to health care provisions (Gracey and King, 2009; Hotz, 2014b). Indigenous communities of the Gran Chaco show consistently high seroprevalence of human T. cruzi infection, ranging from 12 to 83% with local variation (Canese and Brice, 1977; Rojas de Arias et al. 1993; Moretti et al. 2010; Samuels et al. 2013).

Although the success of vector control interventions in some areas of the Chaco has substantially reduced disease incidence, the main challenge is the long-term sustainability, and in particular, entomological surveillance. Reinfestation of treated dwellings, when the residual effect of insecticides decreases, is a common feature especially in areas with peridomestic vectors and/or reinvasion by secondary vectors from the sylvatic environment (Provecho et al. 2014; Gaspe et al. 2015). Other obstacles adversely affecting control in the Chaco include the low efficacy of pyrethroid insecticide spraying on poorly constructed peridomestic structures in this region (Gürtler et al. 2007; Cécere et al. 2013). Of further concern, is the appearance of Triatoma infestans populations resistant to pyrethroid insecticides in localities of northern Argentina and southern Bolivia (Lardeux et al. 2010; Gurevitz et al. 2012). Sylvatic populations of T. infestans have been identified in the Chaco and pose a potential risk of reinvasion (Noireau et al. 1997a; Ceballos et al. 2011; Quisberth et al. 2011; Rolón et al. 2011) as do secondary vectors, including Triatoma sordida (Almeida et al. 2000; Damborsky et al. 2001; Feliciangeli et al. 2003; Lauricella et al. 2005); additionally sylvatic mammals are potential reservoirs of infection, and all of these factors may confound effective control. This study ascertains the different triatomine species present in the region, the mammal species infected and the associated circulating T. cruzi DTUs. Through a better understanding of the T. cruzi transmission dynamics we aim to improve control strategies.

MATERIALS AND METHODS

Fieldwork

Fieldwork collections were performed from 2002 to 2008, with the objective of obtaining and genotyping new isolates of T. cruzi from sylvatic bugs and sylvatic mammals. Isolates obtained in previous surveys (Yeo et al. 2005; Llewellyn et al. 2009, Rojas de Arias et al., manuscript in preparation) were also included to generate a more detailed picture of the distribution of T. cruzi DTUs in the Chaco region.

Study area

The study area encompasses the Paraguayan Chaco (western region), Bolivian Chaco (southern region) and also three further Paraguayan localities 250 km northeast of Asunción (San Pedro, San Alfredo and Aguaapey), within the Department of San Pedro. Study areas are shown in Fig. 1. In total, data were acquired from 28 localities, 24 Paraguayan and four Bolivian. Of the 24 Paraguayan localities, 21 were situated in the Paraguayan Chaco spanning three departments (Boquerón, Presidente Hayes and Alto Paraguay). Within the Department of Boquerón 12 localities were studied: Betania, Campo Loro, Campo Nuevo, Campo Salado, Cesarea, Galilea, Jerico, Campo Alegre, Casuarina, Jotoisha, Tiberia and Samaria. Within the Department of Presidente Hayes a further eight localities were included: Cerrito, Estancia Salazar, 12 de Junio, 20 de abril, Campo Largo, 10 Leguas, Fischat and Jope. Lastly, the one locality from the third department was Don Anibal ranch. These aforementioned localities have been under epidemiological surveillance by the National Program Control of Chagas since 2001. Further details regarding localization, ethnic group and estimated population size of these communities is shown in Table A1 (Appendix A). The remaining three localities lie within the Department of San Pedro (Fig. 1), in the Southeast Chaco: San Pedro, San Alfredo and Aguaapey. Localities from the Bolivian Chaco region, San Antonio, Mora, Cuatro Cañadas and Gutiérrez were all from Santa Cruz Department.

Indigenous communities consist of nomadic hunters, gatherers and fishermen with some groups of sedentary farmers from distinct linguistic groups (Rojas de Arias, 2003). Dwellings are typically of low quality, walls constructed of wattle and brick,
adobe or palm trunk, soil floor with straw, and palm leaves or tin as roofing material. Villages are located in peripheral areas surrounding Mennonite towns. Domestic animals consist primarily of dogs and chickens. Further to the Northeast and in the Bolivian Chaco land use is predominantly for cattle farming and agriculture. To the east of the Paraguayan Chaco, the Department of San Pedro is characterized by more abundant vegetation and intensive agricultural land use.

Collection of triatomine bugs and sylvatic mammals

Each locality was visited only once. Most of the collection of domiciliary and peridomestic triatomines was made by manual searches (1·0 person hour per house) (Gürtler et al. 1998). Domestic areas inspected included walls, interior side of roofs, furniture and bedding. Peridomestic searches encompassed chicken coups, wood piles and other peridomestic structures (Gurevitz et al. 2011). In Campo Loro, Estancia Salazar and Fischat localities domestic bugs were obtained by the collection of local inhabitants. Sylvatic triatomines were collected by the use of live-bait Noireau traps (Noireau et al. 2000), manual dissection of natural ecotopes (bird nests, fallen trees and scrub) and light traps (Vázquez-Prokopec et al. 2006). Light traps were left overnight and checked in the morning. Captured bugs were placed in labelled containers, the developmental stage noted and identified to the level species by trained personel according to Lent and Wygodzinsky (1979). The captured nymphs were raised in laboratory conditions until they reached the adult stage to confirm the species. Sylvatic mammals were captured by the use of
Table 1. DTU discrimination based on PCR amplification products (bp)\(^a\)

| PCR reaction | TcI | TcII | TcIII | TcIV | TcV | TcVI | DTU identification |
|--------------|-----|------|-------|------|-----|------|-------------------|
| 24S\(\alpha\) rRNA | 110 | 125 | 110 | 120\(^b\) | 110/125 | 125 | TcIV, TcV |
| 18S rRNA | 160 | 165 | 165 | 155 | 165 | 165 | TcI, TcIV |
| Mini-exon | 350 | 300 | 250\(^e\) or none\(^e\) | 400\(^e\) or none\(^d\) | 300 | 300 | TcI, TcIV, TcIII |
| RFLP-PCR\(^c\) (HSP60/EcoRV) | 462/462/314/148 | 462 | 462/314/148 | 462/314/148 | 462/314/148 | TcIII\(^f\) |

\(^a\) According to Yeo et al. (2005) and Lewis et al. (2009).
\(^b\) Brisse et al. (2000) reported bands of 125 bp in one strain (Saimiri 3) and of 130 bp for three strains of North American origin. Band of 117 bp was reported by Kawashita et al. (2001).
\(^c\) According to Brisse et al. (2000). They reported a product a low intensity band of 300 bp in two strains (M6241 cl6 and M5631 cl5).
\(^d\) According to Brisse et al. (2000).
\(^e\) According to Westenberger et al. (2005) and Lewis et al. (2009).
\(^f\) Differentiates between TcII and TcVI.

Isolation and characterization of trypanosomes

Trypanosomes were obtained from animals via xenodiagnosis and from triatomines by haemoculture as previously described (Miles, 1993). Mammals were first anaesthetized by intramuscular injection using ketamine (Holliday-Scott\(^e\), 50–80 mg kg\(^{-1}\) body weight). Animal-handling procedures were in accordance with the American Society of Mammalogists (Sikes and Gannon, 2011). To exclude the presence of mixed infection biological clones of Trypanosoma cruzi were obtained by direct culture of infected triatominic feces, onto solid medium agar plates, as described previously by Yeo et al. (2007), five clones of each isolate were expanded in culture and DNA extracted with a kit (QIAEASYTM). Primers and reaction conditions are described in Table B1 (Appendix B). A panel of reference strains, encompassing the known DTUs, was obtained from the London School of Hygiene and Tropical Medicine cryobank repository and consisted of X10 Clone I (DTU TcI), Esmeralda-cl3 (DTU TcII), Arma 13 (DTU TcIII), CAN III (DTU TcIV), SC43 (DTU TcV) and CL Brener (DTU TcVI).

RESULTS

Triatomines

A total of 1044 triatominic bugs were included in the current study, 1037 from Paraguay and seven from the Bolivian Chaco. Triatoma infestans (\(n = 715\)) was found in both domestic and peridomestic environments (\(n = 245\) in domestic; \(n = 470\) in peridomestic) and T. sordida (\(n = 203\)) only in peridomestic environments (Table 2). Adults, fourth- and fifth-instar nymphs were collected from both species in both areas. In Betania, Campo Salado, Galilea both species shared the same niche in chicken coops. In the localities of Campo Nuevo, Cesarea and Samaria only T. sordida was present, while in Estancia Salazar only T. infestans was found. Although T. infestans was collected from both peridomestic and domestic areas, only domestic specimens were microscopically positive for T. cruzi. Thus, 55 (5.4%) of T. infestans were positive, including adults (\(n = 41\)), fourth-instar (\(n = 10\)) and fifth-instar (\(n = 4\)) nymphs. Positive triatomines were from the localities of Jerico (\(n = 31\)), Galilea (\(n = 2\)), Betania (\(n = 1\)), Campo Loro (\(n = 5\)), Estancia Salazar (\(n = 8\)), Jope (\(n = 5\)) and Fischat (\(n = 3\)). In the sylvatic area, 115 of adults (\(n = 107\)) and nymphs (\(n = 8\)) of Triatoma guasayana, three adults of T. sordida, and one female of Triatoma platensis were captured from the localities of Campo Loro, Don Anibal ranch and Betania respectively and they were microscopically negative. Triatomines obtained from Bolivian Chaco by the cooperation of local inhabitants included seven collapsible ‘live-traps’ including Sherman (H.B. Sherman Trap, Inc., Tallahassee, FL) and Tomahawk (Tomahawk Live Trap Co., WI), baited with a mixture of peanut butter, ripe banana and oats. Ten traps per night were set at approximately 10 m intervals on animal trails, near burrow entrances, in dense scrub or close to fallen trees. Traps were left in situ for 3 days, set at sunset and examined at dawn, where applicable local hunters were hired to collect live mammals. All specimens captured were sexed, identified to the species level (Neris et al. 2002), and released unharmed after processing (see below).
Table 2. Summary table: species captured, location, ecotopes, number of positives and DTUs observed

| Country         | Department | Locality   | Species                  | Total captured | Ecotope                | Positive |
|-----------------|------------|------------|--------------------------|----------------|------------------------|----------|
| Paraguay        | Boquerón   | Betania    | Trypanosoma cruzi        | 97             | Domestic 15  Peridomestic | 1^a 1    |
|                 |            |            | Triatoma infestans       | 82             | 1                      | –        |
|                 |            |            | Triatoma sordida         | 18             | 1                      | –        |
|                 |            |            | Trytophagus platensis    | 1              | –                      | –        |
| Campo Loro      |            |            | Trypanosoma cruzi        | 5              | Domestic^b | 1^c 5        |
|                 |            |            | Trytophagus platensis    | 10             | 2                      | –        |
|                 |            |            | Dasypus novemcinctus     | 2              | 1^d 2                  | –        |
|                 |            |            | Euphractus sexcinctus    | 18             | 1                      | 1        |
|                 |            |            | Chaetophractus spp.      | 41             | 1                      | 1        |
| Campo Nuevo     |            |            | Trytophagus platensis    | 39             | Peridomestic           | –        |
| Campo Salado    |            |            | Trytophagus platensis    | 76             | Peridomestic           | –        |
| Cesarea         |            |            | Trytophagus platensis    | 40             | Peridomestic           | –        |
| Galilea         |            |            | Trytophagus platensis    | 188            | Peridomestic 2  Domestic | 2^a 2    |
|                 |            |            | Triatoma sordida         | 69             | Peridomestic           | –        |
| Jerico          |            |            | Trytophagus platensis    | 230            | Peridomestic 207  Domestic | 31^a 26 1 4 |
| Campo Alegre    |            |            | Trytophagus platensis    | 3              | –                      | 3        |
| Casuarinae      |            |            | Trytophagus platensis    | 1              | –                      | 1        |
| Jotoisha        |            |            | Trytophagus platensis    | 1              | –                      | 8        |
| Samaria         |            |            | Trytophagus Platensis    | 32             | Peridomestic           | –        |
| Tiberia         |            |            | Trytophagus Platensis    | –              | Domestic               | 1        |
| Presidente Hayes| Estancia Salazar | Trytophagus Platensis | 111            | 103 Peridomestic 8  Domestic | 8^a 4 4    |
|                 |            |            | Trytophagus Platensis    | –              | Domestic               | 16 21    |
|                 |            |            | Trytophagus Platensis    | 20 de abril   | –                      | 6        |
|                 |            |            | Trytophagus Platensis    | 10 Leguaes    | –                      | 1 6      |
|                 |            |            | Trytophagus Platensis    | Fischat       | –                      | 2        |
|                 |            |            | Trytophagus Platensis    | 5             | Domestic               | 3 3^c 1 1 |
|                 |            |            | Trytophagus Platensis    | –              | Domestic               | 5 2 2 1 1 |
sylvatic adult bugs, *T. sordida* (one specimen) and *T. guasayana* (six specimens). One *T. guasayana* specimen from the locality of Mora was positive.

**Sylvatic mammals**

A total of 138 mammals were included in the current study, 26 from the Bolivian Chaco and 112 from Paraguayan localities. From Bolivia animals representing six species were examined, *Euphractus sexcinctus* (*n* = 7), *Dasypus novemcinctus* (*n* = 9), *Chaetophractus* spp. (*n* = 6), *Tamandua tetradactyla* (anteaters, *n* = 1), *Dasyprocta* spp. (agutí, *n* = 2) and *Nasua nasua* (coati, *n* = 1). Eight animals (30%) were found to be infected by haemoculture and/or xenodiagnosis: five *D. novemcinctus*, two *E. sexcinctus* and one *Chaetophractus* spp. from Gutierrez and Mora localities.

From Paraguayan localities a total of 112 sylvatic animals of five species were captured and examined. In the Chaco localities, 84 mammals included four different armadillo species: *E. sexcinctus* (*n* = 20), *D. novemcinctus* (*n* = 5), *Cabassous* spp. (*n* = 1) and *Tamandua tetradactyla* (anteaters, *n* = 1). Six animals (7%) from Campo Loro and Don Aníbal ranch were infected: two *E. sexcinctus*, two *Chaetophractus* spp. and two *D. novemcinctus*. All the specimens captured in Cerrito were negative by microscopy, xenodiagnosis and haemoculture. Twenty-eight animals were captured from San Pedro. They included *D. novemcinctus* (*n* = 14) and *Didelphis albiventris* (opossum, *n* = 14). Three animals (11%) from Colonia San Alfredo and Aguapey, one armadillo and two opossums, were infected.

**Trypanosome isolates**

A total of 166 *T. cruzi* isolates were included in this study, consisting of 63 new field isolates and a further 103 obtained from previous collections, as described below. Table 2 summarizes the origins of the isolates.

Sixty-three isolates were genotyped to DTU; ten from eight triatomines and two *Didelphis* spp., could not be maintained in culture and were excluded. The isolates from previous collections (Table 2) included 12 that had been genotyped, six from domestic *T. infestans* and six from sylvatic armadillos from the Chaco region of Paraguay (Yeo et al. 2005). Eighty-three isolates originated from domestic *T. infestans* (Rojas de Arias et al., in preparation). Eight isolates were from sylvatic armadillos in Bolivia (Llewellyn et al. 2009).

**Characterization of trypanosome isolates**

Amplicon sizes obtained with new trypanosome isolates were as expected for the corresponding DTU, according to previous surveys (Yeo et al. 2005; Lewis et al. 2009). Examples for PCR–RLFP...
HSP60/EcoRV that differentiate between TcIII, TcII and TcVI are shown in Fig. 2. Domestic T. infestans (n=136) were infected with TcII, TcIII, TcV and TcVI. The most common DTU was TcV (64%), followed by TcVI (28%), TcII (6.6%) and TcIII (1.5%). Twenty-nine sylvatic isolates examined from different armadillo species and one T. guasayana (Bolivia) showed an amplicon profile corresponding to DTU TcIII. Five biological clones from each one of the 63 new field isolates were genotyped to detected mixed infections, but none were found.

Spatial distribution of DTUs
The locality with the most T. cruzi diversity was Jope, with all 4 DTUs circulating in the domestic transmission cycle, followed by Jerico, Casuarina and Fischat with three different DTUs. DTU distribution in the study areas is shown in Fig. 1 and Table 2. Within the domestic transmission cycle nine isolates of TcII were identified, all originated from T. infestans from six Chaco localities: Campo Loro (n=1), Tiberia (n=1), Casuarina (n=1), Jope (n=2), Campo Largo (n=1) and Fischat (n=3). DTU TcV was the most widely dispersed DTU in domestic areas, observed in 11 localities, which were Campo Loro (n=5), Campo Largo (n=6), Jerico (n=26), 12 de Junio (n=16), Casuarina (n=18), Fischat (n=1), Jope (n=1), Galilea (n=2), Campo Alegre (n=3), Jotoisha (n=8) and Betania (n=1). Lastly, TcVI was identified in domestic areas of nine localities: Jerico (n=1), Casuarina (n=1), Tiberia (n=1), Estancia Salazar (n=4), 12 de Junio (n=21), 20 de abril (n=6), 10 Leguas (n=2), Fischat (n=1) and Jope (n=1).

TcIII was predominant among sylvatic isolates and widespread in different armadillo species, including D. novemcinctus (n=20), E. sexcinctus (n=6) and Chaetophractus spp. (n=3) from both Paraguay and Bolivia. TcIII was rarely observed in the domestic environment, being found in only two T. infestans specimens from the Jope locality of the Paraguayan Chaco. A single sylvatic specimen of T. guasayana from Mora locality (Bolivia) also harboured TcIII.

DISCUSSION
Triatomines and mammal species
Triatomines. Four triatomine species were found in the current study: T. infestans (n=715), T. sordida (n=203), T. guasayana (n=115) and T. platensis (n=1). Triatoma infestans was the only species found in the domestic environment and there was no evidence of domiciliation by T. guasayana and T. sordida. The latter two species were found in peri-domestic and/or sylvatic areas. A single specimen of sylvatic T. platensis was captured. This presence of T. infestans in human dwellings (245 specimens) and peridomestic habitats (470 specimens) confirms
that it is the primary vector of human *T. cruzi* infection in the Paraguayan Chaco (Rojas de Arias et al. 1990, 1993, 2011; Zelada et al. 1998; Acevedo et al. 2002). The overall prevalence of *T. cruzi* infection in captured triatomines was 5.4%, and all those infected in Paraguay were domestic *T. infestans*. One explanation is peridomestic *T. infestans* feeding on avian blood in chicken coops, and birds cannot maintain *T. cruzi* infection (Miles et al. 2003). Considering only domestic *T. infestans* the infection rate is 22.4%, representing a substantial risk of transmission to both humans and domestic animals. These findings accord with previous studies in the Paraguayan Chaco, which report human seroprevalence ranging from 12 to 83%, and house infestation rates from 26 to 100% (Canese and Brice, 1977; Chapman et al. 1984; Rojas de Arias et al. 1993, 2003). Thus, the Chaco region is the most highly endemic for Chagas disease in Paraguay, primarily among native Amerindians of the low socioeconomic status.

In peridomestic environments, two triatomines species were found (*T. infestans* and *T. sordida*). *Triatoma infestans* in peridomestic areas poses a potential risk of re-infestation of buildings if surveillance measures are not continuous. Although peridomestic areas are also insecticide sprayed as part of the national control programme, the residual effect is lower than in the domicile because of exposure to climatic conditions. As a consequence this makes peridomestic bugs more difficult to control, requiring that spraying and surveillance to be more frequent. *Triatoma sordida* is widely distributed throughout Central Brazil, Eastern and Central Bolivia, the Chaco region of Paraguay and north-western Argentina where it occurs primarily in the sylvatic environment (Lent and Wygodzinsky, 1979; Diotaiuti et al. 1995). In the Bolivian Chaco, two putative cryptic species belonging to *T. sordida* complex, named groups 1 and 2, respectively, were recognized circulating in sympathy, using multilocus enzyme electrophoresis (Noireau et al. 1998). This species shows great capacity of adaptation to peridomestic sites, especially in association with chickens (Macchiaverna et al. 2015). In the current study, all specimens (*n* = 203) were negative for flagellates probably because their principal food sources are avian. A primary domiciliation by *T. sordida* in the Chaco region was described in localities of Velasco Province, Department of Santa Cruz (Bolivia), where 16.2% of bugs were found infected by *T. cruzi*, although the probability of transmission to humans was considered low (Noireau et al. 1997b; Brehier et al. 1998). Three species of sylvatic triatomine were found: *T. guasayana* (*n* = 115), *T. sordida* (*n* = 3) and *T. patensii* (*n* = 1). Their typical ecotopes were fallen trees and dense shrubs, where they were captured using Noireau traps. *Triatoma guasayana* was an active flyer seeking out potential hosts, with most flight activity occurring just after sunset. Both *T. guasayana* and *T. sordida* have been implicated as sylvatic vectors of *T. cruzi* in parts of the dry Chaco region (Wisnivesky-Colli et al. 1997; Vezzani et al. 2001). In Paraguayan localities, both species were frequently observed near and around households, especially the adults, which have a great capacity for flight (Yeo et al. 2005; Rolón et al. 2011).

Because of these characteristics and their ability to colonize man made structures, they are candidate as secondary vectors. All our sylvatic *T. guasayana* and *T. sordida* captured in Paraguay were not infected with *T. cruzi*, apart from a single sylvatic Bolivian *T. guasayana*. However, infected *T. guasayana* and *T. sordida* have previously been reported in the Argentineen and Bolivian Chaco (Noireau et al. 2000; Bar et al. 2002; Lauricella et al. 2005; Ceballos et al. 2009) with average infection rates of 13.3 and 9.1%, respectively. In the Argentineen Chaco, sylvatic *T. sordida* have been reported with high infection rates (38.5%; Bar and Wisnivesky-Colli, 2001; Bar et al. 2002).

Here we did not find *T. infestans* in the sylvatic ecotope. However, sylvatic ‘dark morph’ *T. infestans* have been reported in the Chaco region of Bolivia, in nests of *Myiopsitta monachus* (parrot), in bromeliads and hollows of live trees in several localities (Noireau et al. 1997a, b; Brehier et al. 2012; Waleckx et al. 2012), in the Argentineen Chaco (Ceballos et al. 2009, 2011) and Chile (Bacigalupo et al. 2006). Prevalence of *T. cruzi* infection is markedly lower in such ‘dark morph’ forms from the Chaco region with prevalence of between 2.5 and 12.5% (Noireau et al. 2000; Brehier et al. 2012; Waleckx et al. 2012) or zero (Ceballos et al. 2009, 2011) probably due that avian blood is the more often source of food. A few surveys have previously reported putative sylvatic populations of *T. infestans* in the Paraguayan Chaco, although they were also presumed attributable to dispersed peridomiliary populations (Velázquez and González, 1959; Ussing et al. 1966; Yeo et al. 2005). More recently putative sylvatic colonies were discovered using a trained dog (Rolón et al. 2011), and these bugs were found 3 km from infested houses. It is significant that this species is capable of surviving in sylvatic ecotopes in at least in some regions of the Paraguayan Chaco. Further research is needed to establish the risk of reinvasion from such sylvatic populations of *T. infestans*.

**Mammals.** Eight species of mammals belonging to five different orders were captured in the study area. The overall prevalence of infection by *T. cruzi* was 12.3% (17/138), although this percentage varied according to the genus. Armadillos were the most common species captured in both regions of Paraguay (Chaco and San Pedro Departments) and from different localities in Bolivia. In two recent
surveys performed in the humid Argentinean Chaco, marsupials and rodents together with armadillos were the most frequently captured species (Alvarado-Otegui et al. 2012; Orozco et al. 2013). The scarce number of marsupials captured and none for rodents in our study is probably related to the environment, since most of our successful collections were from the dry zone of the Chaco. The highest rate of infection was observed in *D. novemcinctus* (28.5%) followed by *E. sexcinctus* (14.8%) and *Chaetophractus* spp. (4.7%). In previous surveys in the same area, infection in armadillos ranged from 3 to 63%, with the highest prevalence in the *Dasypus* (Yeo et al. 2005; Llewellyn et al. 2009). *Dasypus novemcinctus* and the other armadillo species are commonly hunted by the inhabitants of rural communities for food or for handicraft products, and they may be kept alive for several days before being used. Thus, infected armadillos pose a risk for bringing sylvatic *T. cruzi* into the domestic habitat. The triatomine vectors involved in sylvatic transmission cycles in the Chaco region remain uncertain. Members of the genus *Panstrongylus* were reported associated with armadillo burrows in Brazil (Grisard et al. 2000), Venezuela (Llewellyn et al. 2009) and Argentina (Alvarado-Otegui et al. 2012). Our finding of one infected sylvatic *T. guasayana* in fallen trees in Bolivia could suggest some role in sylvatic transmission. The omnivorous behaviour of some mammal species also may contribute to their acquisition of infection (Rabinovich et al. 2001). The prevalence of *T. cruzi* infection in *Chaetophractus* spp. was lower than the other armadillos. Although this species construct their own burrows, they are nomadic and rarely use the same burrow twice, and thus unlikely to become infected with triatomines. Three other mammal species: *T. tetradactyla* (ant-eater), *Dasypus nova* spp. (*aguti*) and *N. nasua* (coati) from Bolivian localities were not infected with *T. cruzi*. Natural infection of *T. tetradactyla* by *T. cruzi* has been reported in Brazil (Miles et al. 1981; Bento et al. 1992; Fernandes et al. 1999) and Colombia (Ramirez et al. 2011). In addition, anteaters and coati are the known hosts of *T. rangeli* (Miles et al. 1983; Deree et al. 2001).

Two *D. albiventris* of 14 captured (14.2%) from the Department of San Pedro were infected with *T. cruzi*, although isolates were not genotyped. These marsupials are usually found in humid areas, so the dry expanse of some Chaco zones may not be suitable for them. They are frequently observed in close proximity to human populations, and high *T. cruzi* infection rates have been found in Brazil (21.9 and 45.2% prevalence; Grisard et al. 2000) and in the humid Chaco of Argentina (36 and 38% prevalence; Alvarado-Otegui et al. 2012; Orozco et al. 2013). In San Pedro Department, *T. cruzi* has also been found in the terrestrial opossum *Monodelphis domestica* (Yeo et al. 2005). Further studies are needed to understand fully the role of marsupials in transmission of *T. cruzi* in Paraguay.

**Host–vector of *T. cruzi* genotypes in the Paraguayan Chaco.** Ours is the most comprehensive survey of *T. cruzi* genotypes in the Paraguayan Chaco region, providing new insight into the transmission dynamics and dispersion among domestic and sylvatic cycles. TcII, TcIII, TcV and TcVI were circulating in the region, with the hybrids TcV and TcVI being most frequently found, supporting earlier observations (Yeo et al. 2005; Lauthier et al. 2012; Maffey et al. 2012; Pérez et al. 2013). TcV and TcVI were predominant and the most dispersed, and found solely infecting *T. infestans* in the domestic cycle, also in agreement with previous surveys (Chapman et al. 1984; Acosta et al. 2001; Yeo et al. 2005). TcV and TcVI were reported in domestic *T. infestans* in the Bolivian Chaco (Pérez et al. 2013), in domestic and peridomestic triatomines (*T. infestans* and *T. sordida*) and domestic dogs and cats in the Argentineen Chaco (Maffey et al. 2012; Enríquez et al. 2013). Thus, TcV and TcVI constitute the largest current threat to human health, and have been associated with severe chronic manifestations of Chagas disease in the southern Cone countries (Corrales et al. 2009; Cura et al. 2012; Vicco et al. 2012; Lucero et al. 2016). TcV and TcVI are infrequently reported in sylvatic cycles: TcV has been observed in one sylvatic *D. novemcinctus* and one *E. sexcinctus* in Paraguay (Yeo et al. 2005), in a rodent (*Octodontomys* spp.), three opossums, two ferrets and one skunk in Argentina (de Luca D’oro et al. 1993; Montamat et al. 1992), and in two sylvatic triatomines (*Triatoma* spp.) from the Bolivian Chaco (M. Llewellyn, unpublished data). There is one record of TcVI in a *D. marsupialis* in the Northeast La Paz (the Jungas and Alto Beni regions) in Bolivia (Valette et al. 1988). It has been suggested that the domestic predominance of TcV and TcVI may be due to their recent anthropogenic origin and rapid clonal dissemination with *T. infestans* and human migration (Lewis et al. 2011). The occurrence of sylvatic TcV and TcVI in other regions, such as the Atlantic forest, remains to be fully explored.

TcII was found only in domestic *T. infestans*, in agreement with previous surveys in the Paraguayan Chaco, where it is also associated within human infections (Acosta et al. 2001; Yeo et al. 2005), although in lower frequency than the TcV and TcVI hybrids. TcII has been detected in single triatomines carrying mixed infection with TcVI (Yeo et al. 2007), and the presence of TcII may have been underestimated as discriminatory markers have not been applied. Like TcV and TcVI, TcII rarely been reported in sylvatic cycles, although this may reflect limited research. Recently, TcII
was reported infecting one sylvatic *T. infestans* in the Bolivian Chaco (Waleckx et al. 2012). Likewise, this DTU has been reported from one monkey (Acosta et al. 2016) and one *E. sexcinctus*, in Paraguay (Yeo et al. 2005) and from sylvatic mammals in Brazil (Fernandes et al. 1999; Bhattacharyya et al. 2015; Lisboa et al. 2015). Sylvatic TcII reservoirs are of particular interest as it is considered to be ancient (Westenberger et al. 2005; de Freitas et al. 2006).

A striking predominance of TcIII was apparent in sylvatic isolates. Twenty-nine sylvatic armadillos from Paraguay (both regions) and Bolivia, one sylvatic *T. guasayana* (from Bolivia) and two domestic *T. infestans* (from Paraguay) harboured TcIII. TcIII is frequently and widely found in sylvatic habitats with armadillos, particularly the genus *Dasypus* (Yeo et al. 2005; Llewellyn et al. 2009; Morocoima et al. 2012). Armadillos infected with TcIII were also reported in Colombia (Saravia et al. 1987), Venezuela (Llewellyn et al. 2009; Morocoima et al. 2012), Bolivia (Llewellyn et al. 2009), Brazil (Lisboa et al. 2009; Marcili et al. 2009b) and Argentina (Alvarado-Otegui et al. 2012; Orozco et al. 2013). In San Pedro (Paraguay), armadillos and one specimen of *M. domestica* (opossum) were infected previously with TcIII (Yeo et al. 2005). One sylvatic *T. guasayana* from the Bolivian Chaco carried TcIII, presumably acquired by feeding on an armadillo; this is the first report of TcIII in *T. guasayana* in Bolivia. *Triatoma guasayana* is frequently found near houses, attracted by light and CO₂, may therefore introduce TcIII into the domestic cycle. This DTU has also been isolated from terrestrial sylvatic triatomines collected, such as *P. geniculatus, T. rubroviria, T. brasiliensis* and *T. vitriceps* in Brazil (Póvoa et al. 1984; Martins et al. 2008; Santos-Mallet et al. 2008; Lisboa et al. 2009) and from *Panstrongylus spp.* associated with a burrow of *D. novemcinctus* in Venezuela (Llewellyn et al. 2009).

Two domestic *T. infestans* from the Chaco region of Paraguay harboured TcIII. Previously in the same region TcIII isolates were obtained from domestic dogs (Chapman et al. 1984) and from sylvatic armadillos (Yeo et al. 2005), suggesting leaky separation between domestic and sylvatic cycles. Dogs are commonly used for hunting of armadillos in the Chaco region, and dogs may thus introduce TcIII into the domestic transmission cycles, but TcIII has so far not been isolated from human cases of Chagas disease in the Chaco region. In contrast, in the eastern region of Paraguay (Cordillera and Paraguari Departments), using amplification products of the 24Sα tRNA, mini-exon and hybridization, TcIII was reported in human cases and domestic *T. infestans* (del Puerto et al. 2010) as well as from domestic and peridomestic *T. sordida* from Concepción Department (Sánchez et al. 2012); this interesting and surprising finding merits follow-up analyses.

Notably, TcI was absent from this survey. TcI has predominantly been found associated with arboreal marsupials, especially the *Didelphis* opossum throughout the Americas but also with rodents and other sylvatic mammals (Yeo et al. 2005; Messenger et al. 2015). Records of the presence and distribution of TcI in Paraguay are scarce. It has been identified in the direct analysis of feces of domestic *T. infestans* from the Chaco and eastern region (Cura et al. 2010), in samples from domestic *T. sordida* in Concepción Department (Sánchez et al. 2012), and in one human case from the Chaco region in a mixed infection with TcII (Rioso et al. 2011). Unfortunately, isolates were not obtained from the two infected opossums from San Pedro Department. TcIV was not found among our many Chaco region isolates, but it has been reported by direct analysis of the intestinal contents of domestic and peridomestic *T. sordida* captured in Concepción Department (eastern region) (Sánchez et al. 2012), although additional analyses are required to confirm this observation.

Biological clones analysed in this study produced similar profiles to the original isolates with the combination of PCR techniques used in this study. The cloning technique on solid media has proven to be useful for discriminating mixed infections in *T. cruzi* reservoirs (Yeo et al. 2007), especially when a variety of DTUs are circulating sympatrically in the same area.

In summary, the distribution and the high prevalence of TcII, TcV and TcVI in domestic transmission cycles shows the remarkable diversity of *T. cruzi* in the Chaco region of Paraguay. In eight localities more than one *T. cruzi* DTU was present in the domestic transmission cycle showing the great capacity of *T. infestans* in indigenous communities to harbour a variety of *T. cruzi* populations. Furthermore, there is increasing evidence of interaction between domestic and sylvatic transmission cycles. Especially, TcIII in the Jope locality was found in both transmission cycles, suggesting introduction of TcIII into the domestic cycle. TcIII is known to be highly virulent in mice (Morocoima et al. 2012) and may therefore prove to be an agent of severe human Chagas disease. The abundance and aggressive nature of *T. guasayana* also carries a risk, if it should adapt to colonization of human dwellings.

The data generated here provide a regional baseline for future research and an indication of potential risks for human health. High-resolution analyses, including comparative genomics, will give further insight into *T. cruzi* transmission dynamics, interactions between sylvatic and transmission and molecular genetics, to inform the much needed improved control of Chagas disease in the Gran Chaco region.
ACKNOWLEDGEMENTS

Special thanks to those who gave assistance in the fieldwork in Paraguay, Elvio Benitez, Pedro Recalde, Victoria Bogado (+), Mercedes Bogado, César Zelaya, Cecilio Lezcano and to Carlos Chura from Bolivia, for his help in collecting samples from Bolivia. Thanks to Dr Antonietta Rojas de Arias, Dr Celeste Vega and Dr Mirian Rolón from the Centro para el Desarrollo de la Investigación Científica (CEDIC)/Díaz Gill Medicina Laboratorial/Fundación Moises Bertoni, Asunción, Paraguay for the T. cruzi isolates used in this study.

FINANCIAL SUPPORT

This survey was supported by Wellcome Trust funding, grant number 066806.

REFERENCES

Acevedo, R., Aguiler, M., Miers, R., Velázquez, G. and Canese, J. (2002). First detection of Trypanosoma cruzi in an urban population of the Chaco Paraguayo. Revista Paraguaya de Microbiología 22, 26–37.

Acosta, N., Samudio, M., Lépez, E., Vargas, F., Yaksic, N., Brenière, S. F. and Rojas de Arias, A. (2001). Isozyme profiles of Trypanosoma cruzi stocks from different areas of Paraguay. Memorias do Instituto Oswaldo Cruz 96, 527–533.

Acosta, N., Miret, J., López, E. and Schichini, A. (2016). First report of Sapajus cay naturally infected by Trypanosoma cruzi in San Pedro Department, Paraguay. Revista Brasileira de Parasitologia Veterinária 25, 327–332.

Almeida, C. E., Vinhaes, M. C., de Almeida, J. B., Silveira, A. C. and Costa, J. (2000). Monitoring the domiciliary and peridomiciliary invasion process of Triatoma rubrovaria in the State of Río Grande do Sul, Brazil. Memorias do Instituto Oswaldo Cruz 95, 761–768.

Alvarado-Otegui, J. A., Ceballos, L. A., Orozco, M. M., Enriquez, G. A., Rodríguez, M., Cardiel, M. V., Jhon, W., White, G., Canese, J. and Miles, M. A. (1984). Trypanosoma cruzi from the Paraguayan Chaco: isozyme profiles of strains isolated at Matí-Matí. The Tournal of Protozoology 31, 482–486.

Corrales, R. M., Mora, M. C., Negrette, O. S., Díosque, P., Lucuna, D., Virreira, M., Beneri, S. F. and Basombrio, M. A. (2009). Congenital Chagas disease involves Trypanosoma cruzi sub-lineage IIId in the northern provincial of Salta, Argentina. Infection, Genetics and Evolution 9, 278–282.

Cura, C. I., Mejia-Jaramillo, A. M., Duffy, T., Burgos, J. M., Rodríguez, M., Cardiel, M. V., Kjos, S., Gurgel-Gonçalves, R., Blanchet, D., De Pablos, L. M., Tomasiní, N., da Silva, A., Russomando, G., Cuba, C. A., Aznar, C., Abate, T., Levin, M. J., Ouma, A., Gurtier, R. E., Diquez, P., Solari, A., Triana-Chávez, O. and Schijman, A. G. (2010). Trypanosoma cruzi I genotypes in different geographical regions and transmission cycles based on a microsatellite motif of the intergenic spacer of spliced-leader genes. International Journal for Parasitology 40, 1599–1607.

Cura, C. I., Lucero, R. H., Bish, M. O., Oshiro, E., Fornichelli, L. B., Burgos, J. M., Lejona, S., Brusés, B. L., Hernández, D. O., Severini, G. V., Velázquez, E., Duffy, T., Anch, T., Lattes, R., Alteche, J., Freilí, H., Diez, M., Nagel, C., Vigilano, C., Favaloro, L., Favaloro, R. R., Mercen, D. E., Sosa-Estani, S. and Schijman, A. G. (2012). Trypanosoma cruzi discrete typing units in Chagas disease patients from endemic and non-endemic regions of Argentina. Parasitology 139, 516–521.

Damborsky, M. B., Bar, M. E. and Oscherov, E. B. (2001). Detection of triatomininae (Hemiptera: Reduviidae) in domiciliary and extra-domiciliary ecotopes. Corrientes, Argentina. Cadernos de Saúde Pública 17, 843–849.

de Freitas, J. M., Augusto-Pinto, L., Favaloro, L., Favaloro, R. R., Merino, D. E., Sosa-Estani, S. and Schijman, A. G. (2010). Trypanosoma cruzi discrete typing units in Chagas disease patients from endemic and non-endemic regions of Paraguay. The American Journal of Tropical Medicine and Hygiene 82, 386–390.

de Luca D’Oro, G. M., Cardenal, C. N., Perret, B., Crisci, J. V. and Montamat, E. E. (1993). Genetic structure of Trypanosoma cruzi populations from Argentina estimated from enzyme polymorphism. Parasitology 107, 405–410.

Dereure, J., Barnabé, C., Vie, J. C., Madelefan, F. and Raccut, C. (2001). Trypanosomatidae from wild mammals in the neotropical rainforest of French Guiana. Annals of Tropical Medicine and Parasitology 95, 157–166.

Diotaiuti, L., Pereira, A. S., Loiola, C. F., Fernandes, A. J., Schofeld, J. C., Dujardin, J. P., Dias, J. C. and Chiari, E. (1995). Inter-relation of sylvatic and domestic transmission of Trypanosoma cruzi in areas with and without domestic vectorial transmission in Minas Gerais, Brazil. Memorias do Instituto Oswaldo Cruz 90, 443–448.

Enriquez, G. F., Cardinal, M. V., Orozco, M. M., Lanati, L., Schijman, A. G. and Gurtier, R. E. (2013). Discrete typing units of Trypanosoma cruzi identified in rural dogs and cats in the humid Argentinean Chaco. Parasitology 140, 303–308.
Trypanosoma cruzi discrete typing units in the Paraguayan Chaco

Felicianelli, M.D., Campbell-Lendrum, D., Martínez, C., González, D., Coleman, P. and Davies, C. (2003). Chagas disease control in Venezuela: lessons for the Andean region and beyond. Trends in Parasitology 19, 44–49.

Fernández, O., Mangia, R.H., Lisboa, C.V., Pinho, A.P., Morel, C.M., Zingales, B., Campbell, D.A. and Jansen, A.M. (1999). The complexity of the xyletic cycle of Trypanosoma cruzi in Rio de Janeiro state (Brazil) revealed by the non-transcribed spacer of the mini-exon gene. Parasitology 118, 161–166.

Gürtler, R.E., Provec, Y.M., Piccinali, R.V. and Gürler, R.E. (2015). Where do these bugs come from? Phenotypic structure of Triatominae insects populations after control interventions in the Argentine Chaco. Memorias do Instituto Oswaldo Cruz 110, 310–318.

Gracey, M. and King, M. (2009). Indigenous health part 1: determinants and implications. Australian Journal of Tropical Medicine 101, 165–75.

Grisard, E.C., Carvalho-Pinto, C.J., Scholz, A.F., Toma, H.K., Schlemper, B.R. and Steinadel, M. (2000). Trypanosoma cruzi cruzi infection in Didelphis marsupialis in Santa Catarina and Arvoredo Islands, southern Brazil. Memorias do Instituto Oswaldo Cruz 95, 795–800.

Gurevitz, J.M., Ceballos, L.A., Gasé, M.S., Alvarado-Otegui, J.A., Enriquez, G.F., Kitron, U. and Gürler, R.E. (2011). Factors affecting infestation by Triatoma infestans in a rural area of the humid Chaco in Argentina: a multi-modal inference approach. PLoS Neglected Tropical Diseases 5, e4344.

Gurevitz, J.M., Gasé, M.S., Enriquez, G.F., Vassena, C.V., Alvarado-Otegui, J.A., Provecho, Y.M., Cueto, A.G., Picollo, M.L., Kitron, U. and Gürler, R.E. (2012). Unexpected failures to control Chagas disease vectors with pyrethroid spraying in northern Argentina. Journal of Medical Entomology 49, 1379–1388.

Gürler, R.E. (2009). Sustainability of vector control strategies in the Gran Chaco region: current challenges and possible approaches. Memorias do Instituto Oswaldo Cruz 104, 52–59.

Gürler, R.E., Chuit, R., Céccere, M.C., Castañera, M.B., Cohen, J.E. and Segura, E.L. (1998). Household phenotypic heterogeneity for Trypanosoma cruzi in three rural villages in northwest Argentina: environmental, demographic, and entomological associations. The American Journal of Tropical Medicine and Hygiene 59, 741–749.

Gürler, R.E., Kitron, U., Céccere, M.C., Segura, E.L. and Cohen, J.E. (2005). Strategic control and management of Chagas disease in the Gran Chaco, Argentina. Proceedings of the National Academy of Sciences of the United States of America 104, 16194–16199.

Hotez, P.J. (2014a). Ten global “hotspots” for the neglected tropical diseases. PLoS Neglected Tropical Diseases 8, e2286.

Kawakita, S.Y., Sanson, G.F., Fernandez, O., Zingales, B. and Briones, M.R. (2001). Maximum-likelihood divergence date estimates based on ten molecular sequences suggest two scenarios of Trypanosoma cruzi intraspecific evolution. Molecular Biology and Evolution 18, 2250–2259.

Lardeux, F., Depickère, S., Duchon, S. and Chávez, T. (2015). Insecticide resistance of Triatoma infestans in Triatoma guasayana vector of Chagas disease in Bolivia. The American Journal of Tropical Medicine and Hygiene 92, 437–443.

Messanger, L.A., Miles, M.A. and Bern, C. (2015). Between a bug and a hard place: Trypanosoma cruzi genetic diversity and the clinical outcomes of Chagas disease. Expert Review of Anti-Infective Therapy 13, 995–1029.

Miles, M., Feliciangeli, D. and Rojas de Arias, A. (2003) American tripanosomiasis (Chagas disease) and the role of molecular epidemiology in guiding control strategies. British Medical Journal 326, 1444–1448.

Miles, M.A. (1993). Culturing and biological cloning of Trypanosoma cruzi. In Protocols in Molecular Parasitology, vol. 21 (ed. Hyde, J.E.), pp. 15–28. Springer, London.

Miles, M.A., Póvoa, M.M., De Souza, A.A., Lainson, R., Shaw, J.J. and Ketteridge, D.S. (1981). Chagas’s disease in the Amazon Basin: the distribution of Trypanosoma cruzi in zymodeme 1 and 3 in Pará State, north Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 75, 667–674.

Moretti, E., Castro, I., Franceschi, C. and Basso, B. (2019). Chagas disease: serological and electrocardiographic studies in Wichi and Creole communities of Misión Nueva Pompeya, Chaco, Argentina. Memorias do Instituto Oswaldo Cruz 104, 1251–1259.

Montamat, E.E., De Luca d’Oro, G., Perret, B. and Rivas, C. (1992). Characterization of Trypanosoma cruzi from Argentina by electrophoretic zymogramma. Acta Tropica 51, 173.

Morococha, A., Carrasco, H.J., Boudas, J., Chique, J.D., Herrera, L. and Urdaneta-Morales, S. (2012). Trypanosoma cruzi from armadillos (Dasypus novemcinctus novemcinctus) from Northeastern Venezuela and
situación. XIIa participación comunitaria de etnias indígenas: Una perspectiva de la Investigaciones en Ciencias de la Salud (1990). Rojas de Arias, A., Velázquez de Saldívar, G., Merlo, R., Monzón de Santiago, Chile, Marzo de 2003 (PAHO document), pp. 121
Rojas de Arias, A. lineages in human infection along the endemic area. Trypanosoma cruzi A. and Leguizamón, M. S. Monteón, V. M., Russomando, G., Schijman, A. G., Bottasso, O. Risso, M. G., Sartor, P. A., Burgos, J. M., Briceño, L., 119 Medicine and Hygiene
Oswaldo Cruz Argentine Chaco: an underreported process? Triatoma infestans urban interface and house infestation with Enriquez, G. F., Weinberg, D. and Gürtler, R. E. Provecho, Y. M., Gaspe, M. S., del Pilar Fernández, M., Amazonas and Rondônia, Brazil. Sánchez, H., Acosta, C., Villalba, C., Cécere, C., Marcet, P. A., Burgos, J. M., Briceño, L., 180 the population structure of Trypanosoma cruzi. Genetics
Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. and Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279. Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279. Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279. Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279. Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279. Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279. Velázquez, C. J. y González, G. (1959). Aspectos de la enfermedad de Chagas en el Paraguay. Revista Gauina de Medicina 5, 357–373. Vezzani, D., Schweigmann, N. J., Pietrokovsky, S. M. y Wisnivesky-Colli, C. (2001). Characterization of Triatoma guasayana biotypes in a hardwood forest of Santiago del Este, Argentina. Memorias del Instituto Oswaldo Cruz 96, 459–466. Vicco, M. H., Bontempi, I., Ortiz, S., Solari, A., Bottasso, O. A. and Marcipar, I. (2012). Chronic Chagas disease with cardiodepressive involvement and the TiVI infective form of Trypanosoma cruzi. A case report. Parasitology International 61, 715–727. Valeck, E., Depickère, S., Salas, R., Aliaga, C., Monje, M., Calle, H., Buitrago, R., Noireau, F. and Bourié, S. G. (2006). Seasonal variations in active dispersal of natural populations of Triatoma infestans in rural north-western Argentina. Medical and Veterinary Entomology 20, 272–279.
Trypanosoma cruzi from naturally infected triatomine bugs and from experimentally infected mice by direct plating on a sensitive solid medium. International Journal for Parasitology 37, 111–120.

Zelada, J., Sanabria, V., Canese, A. and Canese, J. (1998). Enfermedad de Chagas en comunidades indígenas del Chaco Paraguayo. Revista Paraguaya de Microbiología 18, 13–17.

Zingales, B., Miles, M. A., Campbell, D. A., Tibayrenc, M., Macedo, A. M., Teixeira, M. M., Schijman, A. G., Llewellyn, M. S., Lages-Silva, E., Machado, C. R., Andrade, S. G. and Sturm, N. R. (2012). The revised Trypanosoma cruzi subspecific nomenclature: rationale, epidemiological relevance and research applications. Infection, Genetics and Evolution 12, 240–253.

APPENDIX A

Table A1. Localities surveyed in the Chaco (Paraguay and Bolivia) and San Pedro Departments

| Country | Department | Locality     | Ethnic group | Latitude  | Longitude  | Inhabitants (approx.) |
|---------|------------|--------------|--------------|-----------|------------|-----------------------|
| Paraguay| Boquerón   | Betania      | Nivaclé      | 22°36'1·6"S 59°48'54"W | 363 |
|         | Boquerón   | Jerico       | Nivaclé      | 22°35'52·71"S 59°48'34·24"W | 153 |
|         | Boquerón   | Cesarea      | Nivaclé      | 22°35'32·59"S 59°49'11·72"W | 144 |
|         | Boquerón   | Samaria      | Nivaclé      | 22°35'55·58"S 59°49'54·41"W | 164 |
|         | Boquerón   | Tiberia      | Nivaclé      | 22°36'41·1"S 59°50'44·93"W | 220 |
|         | Boquerón   | Galilea      | Nivaclé      | 22°35'4·44"S 59°56·46·44"W | 120 |
|         | Boquerón   | Campo Nuevo  | Nivaclé      | 22°34'26·13"S 59°55·34·76"W | 187 |
|         | Boquerón   | Campo Salado | Nivaclé      | 22°34'54·84"S 59°57'00·96"W | 93  |
|         | Boquerón   | Campo Alegre | Lenguá       | 22°51'09"S 60°02'10"W | 348 |
|         | Boquerón   | Casuarina    | Nivaclé      | 22°54'21·63"S 60°00'4·45"W | 274 |
|         | Boquerón   | Jotoisha     | Nivaclé      | 22°26'48·86"S 60°37'11·63"W | 282 |
|         | Boquerón   | Campo Loro   | Ayoreo       | 22°4'48·58"S 59°50'29·19"W | 651 |
|         | Presidente Hayes | 12 de Junio   | Angaité     | 22°56'10"S 59°53'45·4"W | 290 |
|         | Presidente Hayes | 20 de abril  | Nivaclé      | 22°57'57·25"S 59°52·1·1"W | 84  |
|         | Presidente Hayes | Campo Largo  | Nivaclé      | 22°49'44"S 59°54·9·3"W | 506 |
|         | Presidente Hayes | 10 Leguas   | Angaité     | 22°52'8·6"S 59°52·30·3"W | 278 |
|         | Presidente Hayes | Jope        | Nivaclé      | 22°35'55·91"S 59°47'13·03"W | 351 |
|         | Presidente Hayes | Fischat     | Nivaclé      | 23°47'27·64"S 60°47'0·99"W | 731 |
|         | Presidente Hayes | Estancia Salazar | Sanapan | 23°42'20·86"S 59°14'12·09"W | 472 |
|         | San Pedro   | San Pedro    | San Pedro    | 24°11'37·59"S 56°34'45·12"W | 840 |
|         | San Pedro   | San Alfredo  | San Pedro    | 24°34'11·94"S 56°44'3·52"W | 290 |
|         | San Pedro   | Aguapey     | San Pedro    | 24°31'26·82"S 56°47·9·2"W | 220 |
| Bolivia | Santa Cruz | San Antonio | Mora         | 18°27'25·75"S 63°12'29·47"W | 240 |
|         | Santa Cruz | Cuatro Cañadas | 17°30'58·302"S 61°35'58·80"W | 153 |
|         | Santa Cruz | Gutierrez   | 19°26'10·63"S 63°31'43·65"W | 620 |

Data from indigenous communities include: location, ethnic group and estimated population.

a Atlas de comunidades indígenas del Paraguay, http://www.dgeec.gov.py (2012).
### APPENDIX B

**Table B1. Primers used and reaction conditions for each one of the PCR reactions performed**

| PCR reaction | Primers sequence (5′–3′) | Reaction mix (20 µL, total volume) | Reaction conditions | Electrophoretic conditions | Restriction digestion reaction |
|--------------|---------------------------|-------------------------------------|---------------------|---------------------------|-------------------------------|
| **24Sα rRNA** | D71 AAG GTG CGT CGA CAG TGT GG D72 TTT TCA GAA TGGCCG AAC AGT | 12·2 2 0·6 2 1 each one 1 0·2 | 30 cycles: 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C 1 cycle: 5 min at 72 °C | 80 V 0·5× TBE buffer 3% agarose 2 h Hyperladder 5 (Bioline) | Idem to 24Sα rRNA |
| **18S rRNA** | V1 CAA GCGGCT GGG TGG TTA TTC CA V2 TTG AGG GAA GGC ATG ACA CAT GT TC CCC CCC TCC CAG GCC ACA CTG TCI GTG TTC GCCACC TCC TTC GGG CC TC2 CCT GCA GGC ACA CGT GTG TGT G | 12·2 2 0·6 2 1 each one 1 0·2 | Idem to 24Sα rRNA | Idem to 24Sα rRNA |
| **Mini-exon** | CAG GCC ACA CTG TCI GTG TTC GCCACC TCC TTC GGG CC TC2 CCT GCA GGC ACA CGT GTG TGT G | 11·2 2 0·6 2 1 each one 1 0·2 | 27 cycles: 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C 1 cycle: 5 min at 72 °C | 90 V 0·5× TBE buffer 1·5% agarose 90 min Hyperladder 4 (Bioline) | |
| **PCR–RFLP of HSP60** | FWD GTG GTA TGG GTG ACA TGT AC REV CGA GCA GCA GAG CGA AAC AT | 8 2 2 4 1 each one 1 0·2 | 30 cycles: 2 min at 94 °C, 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C 1 cycle: 10 min at 72 °C | 90 V 0·5× TBE buffer 1·5% agarose 1 h Hyperladder 4 (Bioline) | 10 µL PCR product 2 µL 10× buffer 0·2 µL BSA 0·5 µL EcoRV 7·3 µL H2O 37 °C for 4 h |

---

**a** Bioline Ltd. London, UK.

**b** Stained with ethidium bromide and visualized under ultraviolet light.

**c** Bovine serum albumin acetylated 100×