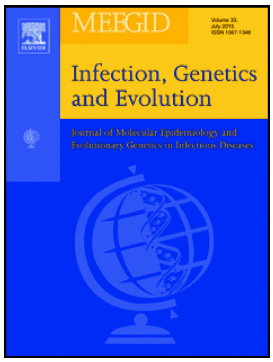

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New human isolates of *Trypanosoma cruzi* confirm the predominance of hybrid lineages in domestic transmission cycle of the Argentinean Chaco

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Abstract

Trypanosoma cruzi, the etiological agent of Chagas disease, was initially classified into 6 Discrete Typing Units (DTUs). The hybrid DTUs TcV and TcVI are the most frequent in domestic transmission cycles throughout the Southern Cone countries of South America. Here, we genotyped parasite isolates from human residents in Pampa del Indio municipality, Chaco, to further characterize the structure of *T. cruzi* populations, and to assess the degree of overlapping between the domestic and sylvatic transmission cycles. Artificial xenodiagnostic tests were performed to blood samples from 125 *T. cruzi*-seropositive people (age range, 3-70 years) who represented 14.3% of all seropositive residents identified. Parasites were obtained from feces of *T. cruzi*-infected *Triatoma infestans* examined 30 or 60 days after blood-feeding, and grown in vitro. The cultured parasites were genotyped by means of two PCR-based protocols. DTUs were determined from 39 (31%) patients residing in 28 dwellings. The only DTUs identified were TcV (92%) and TcVI (8-36%). Households with more than one parasite isolate consistently displayed the same DTU. Further sequencing of a fragment of the TcMK gene from selected samples argue against the occurrence of mixed TcV-TcVI infections in the study population. Sequencing data revealed an unexpected degree of genetic variability within TcV including two apparently robust subgroups of isolates. Our results for human residents confirm the predominance of hybrid lineages (TcV and to a much lesser extent TcVI) and the absence of sylvatic genotypes (TcI and TcIII) in (peri)domestic transmission cycles in the Argentinean Chaco area.

245 words

Keywords: *Trypanosoma cruzi*, DTU, human, molecular epidemiology, isolates, Chaco

Highlights

1) Only hybrid lineages: TcV (92%) and TcVI (8-36%) were detected in human infection.

2) Households with more than one parasite isolate consistently displayed the same DTU.

3) No evidence of overlapping between domestic and sylvatic cycles was detected.

1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is the most important neglected tropical disease (NTD) in Latin America (World Health Organization, 2015). Recent reports estimate that nearly 5-6 million people are infected with *T. cruzi* in the continent, and 70 million are at risk of infection (World Health Organization, 2015). Chagas disease and other NTDs disproportionately affect resource-constrained, vulnerable populations (Hotez et al., 2008), which become trapped in a vicious cycle or "poverty trap" (Ault, 2007).

Trypanosoma cruzi is considered a single species although it has a complex population structure showing great inter-strain genetic diversity. Based on biochemical and genetic markers, the *T. cruzi* taxon was classified into 6 Discrete Typing Units (DTU), TcI-TcVI (Zingales et al., 2009). A potential seventh DTU, termed TcBat, has been mainly found associated with bats (Marcili et al., 2009). Although the evolutionary origin of all DTUs is still under debate, there is an overall consensus that TcV and TcVI are hybrid lineages originated from crossings of TcII and TcIII ancestors (Lewis et al., 2011).

Two vector-borne transmission cycles of *T. cruzi* have been typically characterized, the (peri)domestic and the sylvatic ones, which may overlap to various degrees (Miles et al., 2003). These cycles may include a wide diversity of vectors, hosts, and parasite genotypes; and may have differential relevance in distinct eco-geographic niches (Zingales et al., 2012). Moreover, sylvatic transmission cycles may occur separately in terrestrial and arboreal habitats (Yeo et al., 2005; Noireau et al., 2009). The distribution of *T. cruzi* DTUs in infected people is highly variable among endemic areas across Latin America. Interestingly, the 7 DTUs were found in South America (Brenière et al., 2016)). In Argentina, the most frequent DTU identified in humans is TcV, followed by TcVI. Notably, other DTU prevalent among *T. cruzi*-infected patients in other Latin American countries such as TcI and TcII have rarely been found in Argentina (Bontempi et al., 2016; Bua

et al., 2013; Burgos et al., 2010; Cardinal et al., 2008; Cura et al., 2012; Diez et al., 2010; Diosque et al., 2003; Lucero et al., 2016; Monje-Rumi et al., 2013, 2015). Mixed infections (i.e., more than one DTU identified in a single patient) have been less frequently reported than in Bolivia and Chile (R. Del Puerto et al., 2010; Solari et al., 2001), and were mainly associated with Chagas disease reactivation due to immunosuppression (Burgos et al., 2010).

This study is part of an ongoing research and disease control program implemented since 2007 in the Municipality of Pampa del Indio, Chaco, Argentina, which aims to achieve the sustainable elimination of *T. infestans* from rural dwellings and the interruption of vector-borne transmission. All rural villages within this area have been under sustained vector surveillance and control, and house infestation was kept under 3% district-wide (Gaspe et al., 2015; Gurevitz et al., 2013; Provecho et al., 2017). Several surveys genotyped *T. cruzi* parasites from Pampa del Indio sylvatic and domestic hosts, and vectors. Briefly, TcI was found in *Didelphis albiventris* opossums (Alvarado-Otegui et al., 2012), in the secondary vector *Triatoma sordida* (Macchiaverna et al., 2015; Maffey et al., 2012; Provecho et al., 2014), and rarely in domestic dogs and cats (Enriquez et al., 2014). TcIII was found in different armadillo species (Alvarado-Otegui et al., 2012; Orozco et al., 2013) and in two domestic dogs (Enriquez et al., 2013). TcV and TcVI were the DTUs most frequently found in domestic habitats, including dogs and cats (Enriquez et al., 2013), the primary vector *T. infestans* and *T. sordida* (Maffey et al., 2012). The current study provides the first DTU identifications from human parasite isolates in Pampa del Indio.

Most of the recent literature regarding the distribution of DTUs in humans derives from surveys conducted in urban health centers (including patients from diverse areas) or meta-analyses across the Americas (Brenière et al., 2016; Browne et al., 2017; Lucero et al., 2016; Martinez-Perez et al., 2016; Ramírez et al., 2015; Tavares de Oliveira et al., 2017). Our study sought to obtain and genotype new *T. cruzi* isolates from human residents of a well-defined

endemic rural area to further characterize domestic transmission cycles in the Argentinean Chaco. In doing so, we compared the DTU distribution of human parasite isolates with those recorded locally in other sylvatic and domestic hosts and vectors, and probe into the possible overlap between transmission cycles. Based on the background information described above, we hypothesized that TcV and TcVI would be the main DTUs found in local human residents.

2. Material and Methods

2.1 Study area

Fieldwork was conducted in rural areas of Pampa del Indio Municipality (25º55'S 56º58'W), Chaco Province, Argentina, located at the transition between the Humid and Dry Chaco subregions (Figure 1). Approximately 7,000 people resided in 1,400 dwellings grouped in rural villages in 2013-2016. Half of the residents belonged to the Qom ethnic group, and the remainder were Creoles.

The rural area was initially subdivided into four areas (I-IV); a fifth one was added later to include rural villages situated beyond the district's limits (Figure 1). This study includes parasite samples of residents mainly inhabiting areas II, III and IV, and a minority from area V and periurban sections. All periurban and rural dwellings were georeferenced during triatomine surveys.

2.2 Study population

In cooperation with personnel from the local hospital "Dante Tardelli", we conducted several serosurveys which aimed to achieve a complete enrolment of the study population, regardless of their age, gender or ethnicity, over 2012-2017. Venipuncture and blood extraction was preceded by community meetings held at local primary health-care posts and schools, radio advertisements and school notes to enhance community participation (Sartor et al. 2017). People eligible for the serosurvey were residents older than 9 months of age who provided informed

written consent. Parents or guardians of children younger than 18 years of age provided consent for them.

In total, 3,216 residents from areas II, III and IV, and a few area V or peri-urban residents were examined serologically for *T. cruzi* antibodies using procedures detailed in Sartor et al. (2017). Seropositive individuals below 21 years of age were offered etiological treatment with benznidazole, excluding those who had received it previously, pregnant or lactating women, and patients with renal or liver problems or impaired health condition.

For the current study, we performed artificial xenodiagnosis to a total of 125 patients seropositive for *T. cruzi* prior to the etiologic treatment course. The majority of them (74.4%) were younger than 21 years of age.

2.3 Parasite isolation

Artificial xenodiagnosis tests were performed to *T. cruzi*-seropositive residents. For each test, 20 fourth–instar nymphs of laboratory-reared *T. infestans* (kept unfed for at least 3 weeks) were fed with 3 ml of heparinized blood from each patient using a blood-feeding device (Cardinal et al., 2008). The time elapsed between blood extraction and the onset of feeding was less than 5 min. Bugs feces were analyzed by optical microscopy (400×) 30 and 60 days post-feeding.

Parasites from feces of infected *T. infestans* were cultured in biphasic tubes containing blood-agar and brain-heart-infusion (BHI) as described in Lauricella et al. (2005). *In vitro* cultures were incubated at 28 °C and an aliquot was taken weekly to monitor parasite growth. When a high number of parasites was observed (minimum $1.3x10^6$ parasites/ml), 1 ml of the liquid phase was harvested, boiled between 10-15 min, centrifuged, and the supernatant was stored as the stock solutions from which DTU identification was sought.

2.4 DTU identification

DTUs were identified using two different PCR protocols directed to: 1) the spliced-leader sequence (SL, or "mini-exon"), alpha 24s rDNA, and A10 genomic marker (Burgos et al., 2007), and 2) two nuclear genes: TcSC5D and TcMK according to Cosentino and Agüero (2012) protocol. For the latter, the expected amplicons are 832 bp and 657 bp bands, respectively. When it was not possible to observe the expected band of 657 bp, we incorporated a second round of PCRs using the same primers. These amplicons were digested with three restriction enzymes and the band pattern was observed and compared to control *T. cruzi* DNA stocks from strains belonging to known DTUs as described in Cosentino and Agüero (2012). Negative and positive controls are routinely included during the extraction and PCR steps. PCRs included a master mix negative control, which only includes mixture reagents, and a "DNA loading" negative control, which is performed by adding water instead of the target DNA and is loaded after all DNA samples and positive controls. Nested PCRs also include the negative and positive controls of the first round to rule out potential failures.

We used two different protocols because distinguishing between a single TcV infection and a mixed infection of TcV and TcVI is hardly feasible using either protocol. In Burgos et al. (2007), to differentiate TcV from TcVI, a PCR is performed targeting the 24S rDNA; TcVI strains show a band of 290 bp whereas TcV strains show a 275 bp band. However, some TcV strains display both bands. In Cosentino and Agüero (2012), the amplification product of the TcMK gene from TcV strains, unlike TcVI strains, is sensitive to XhoI restriction. However, because some apparent TcV strains do not present complete XhoI digestions, we cannot rule out mixed infections. To solve this ambiguity, a fragment of the TcMK gene was PCR-amplified and fully sequenced.

2.5 Sequencing

To resolve the occurrence of potentially mixed infections, amplicons obtained for the TcMK gene were sequenced using the same primers as in the amplification step (Macrogen Inc., Seoul,

Korea). In addition to human parasite isolates, four reference strains were subjected to the same sequencing analysis: PAH265 for TcV, ClBrener for TcVI, and two strains obtained from Pampa del Indio dogs, PpID166 and PpID159 identified as TcV and TcVI, respectively (Enriquez et al., 2013). Sequences of TcI strains were excluded from this analysis because they were notoriously different from the rest of the DTUs and no TcI infection was found among the study isolates. Additional TcMK sequences were downloaded from GenBank. The alignment was performed using the Mega 7 program (Kumar et al., 2016). Sequences herein reported are available in Genbank (Accession numbers MH671655-MH671667).

2.6 Ethical statement

The procedures for human serological diagnosis and etiological treatment (Protocol N° TW-01-004) and the study of parasite diversity have been approved by the "Comité de Etica en Investigación Clínica" (Ethics Committee in Clinical Research) of Buenos Aires, Argentina.

3. Results

3.1 Parasite isolation

In total, 125 artificial xenodiagnosis tests from *T. cruzi*-seropositive patients were performed, representing 14.3% of all seropositive residents (n = 852). At least one infected triatomine and parasite isolate was obtained from 39 (31%) patients who lived in 28 dwellings located in rural or peri-urban areas (Table 1). The mean age of patients with successful parasite isolation was 17.4 years (standard deviation: 14.6, range: 3-70 years). Most (62%) isolates came from women. The 39 isolates obtained represented 4.6% of all seropositive persons identified. Thirty-three patients were under 21 years old, which represent 18.8% of the young people eligible for etiological treatment (33 patients with isolates/176 eligible patients).

3.2 DTU identification

Human infection with *T. cruzi* was restricted to hybrid lineages with TcV more frequently found than TcVI (Table 2). Using the Burgos et al. (2007) protocol, 100% of the isolates were classified as TcV, although 90% of them might have been co-infected with TcVI parasites because double bands were observed upon amplification of the 24S rDNA locus. Using the protocol by Cosentino and Agüero (2012), 97% of the isolates tested were classified: 61% were only TcV, 8% only TcVI, and 31% were TcV or TcV + TcVI, because partial XhoI digestion was observed (Table 2). One sample with very low DNA concentration (because of culture failure) failed to amplify the TcSC5D and TcMK genes (Table 1). Neither confirmed mixed infections nor single infections with TcI, TcII, TcIII or TcIV were detected (Table 2). The same DTUs were identified in all households with more than one isolate analyzed (n=7, Table 1), including one house where one isolate was identified as only TcV and another as a potential mixed infection of TcV and TcVI using Burgos et al. (2007) protocol but both isolates were identified as TcV using the Cosentino and Agüero (2012) protocol.

We sequenced a 581 bp- fragment from the TcMK gene in 13 *T. cruzi* isolates, 9 of which belonged to humans (named PpIHuXXX), 2 to dogs (PpIDXXX), and 2 to reference strains. When aligning the obtained sequences with those available in GenBank for TcI-TcVI, 34 polymorphic sites were found. All DTUs were clearly differentiated through analysis of this sequence, except TcIII from TcIV (Table 3). All human isolates sequenced were TcV (Table 3). We sequenced isolates which gave single or double bands at 24S rDNA-PCR, and complete or partial digestion of TcMK gene by XhoI. A sample from each village of the study area was included to cover the geographical range (Supplementary Table S1).

Because TcV and TcVI are hybrid DTUs, 21 of these sites were polymorphic SNPs. Six of the SNPs were non-synonymous mutations. Five sites allowed the differentiation of TcV from TcVI:

sites 51, 83 and 309 distinguished TcVI from the rest of the DTUs; site 264 distinguished TcV (A), TcII and TcVI (W) and TcI, TcIII and TcIV (T), and site 64 differentiated some TcV isolates from other DTUs. In addition, four sites differentiated TcII and TcV from the rest of the strains; these sites were 30, 37, 502 and 555 (Table 3).

Some of the isolates here reported did not present heterozygous sites (e.g. PpIHu18, PpIHu19, PpIHu238, PpIHu273 and PpIHu643), unlike the reference TcV strains and some other isolates (Table 3).

4. Discussion

Our study identified the DTUs circulating in residents of a well-defined rural area in the Argentinean Chaco endemic for Chagas disease. TcV predominated among these new *T. cruzi* isolates obtained from artificial xenodiagnostic tests whereas TcVI was much less frequent, in accordance with the known DTUs distribution in Argentina. Sequencing part of the TcMK gene allowed the confirmation of single TcV infections, unlike the results obtained using other protocols.

TcV and TcVI are hybrid DTUs (Pena et al., 2009; Sturm et al., 2003; Westenberger et al., 2005). Because they are genetically similar and of recent divergence, many protocols fail to distinguish between them (Arenas et al., 2012; Higo et al., 2007; Lewis et al., 2009; Ramírez et al., 2017). The two PCR-based protocols we tested displayed a high concordance, but both were unable to distinguish potentially mixed infections, which required sequencing.

Given the small amount of polymorphisms reported in TcV using microsatellites (Brisse et al., 2000; Lewis et al., 2011; Virreira et al., 2006; Yeo et al., 2011), the finding of two sets of isolates within TcV was completely unexpected at this geographical scale. One set (including the reference strains) presented heterozygosis, whereas the other group had no biallelic sites.

Nevertheless, the absence of biallelic sites may be caused by stochastic mutations in the primer binding sites or by loss of heterozygosity, which was previously proposed for TcV (Brisse et al., 2000; Yeo et al., 2011). Although this result appears to be promising for intra-DTU typing, the fact that only one gene was analyzed favors a more cautious stance. Both nuclear and mitochondrial genes need to be examined to evaluate the robustness of these TcV intra-groups.

One of the strengths of this study is its degree of coverage of a well-studied population: the 39 isolates obtained represented a considerable fraction of the identified *T. cruzi*-seropositive residents and a broad age range (3 to 70 years). Patients were reached out at their home, allowing linking a geographic coordinate to the isolate obtained. When more than one parasite isolate was obtained at the same household, there was a 100% concordance between the DTUs obtained within the house.

One of the major limitations of this study is the possibility of strains or DTUs selection during the multi-step process of parasite isolation. On one hand, we limited our sampling to bloodstream parasite populations, which may not be representative of the full spectrum of *T. cruzi* infecting strains (Macedo and Pena, 1998). Inadvertent selection might also occur due to differences among strains in *in vitro* culture growth (Bosseno et al., 2000) or to differences in strain development within the insect vector used for artificial xenodiagnosis (Buscaglia and Di Noia, 2003). The latter procedure was implemented to increase the sensitivity of our analyses. Although *T. cruzi* DTUs have been successfully identified from whole blood, the probability of successful identification decreases markedly in chronic patients and with increasing age (Bontempi et al., 2016; Burgos et al., 2007; Cura et al., 2012; Lucero et al., 2016). All isolates here reported were from chronic patients, and 25% belonged to people aged ≥ 18 years. Because the protocol proposed by Cosentino and Agüero (2012) targets two single-copy nuclear genes, high parasite concentrations are needed, but these are hardly ever found in chronic patients' blood.

Using the Burgos et al. (2007) protocol, we were able to identify TcI, TcII, TcIII, TcV and TcVI from samples of different host and vector species (Alvarado-Otegui et al., 2012; Argibay et al., 2016; Cardinal et al., 2008; Enriquez et al., 2014, 2013; Macchiaverna et al., 2015; Maffey et al., 2012; Orozco et al., 2013). Therefore, the absence of TcI, TcII and TcIII among the human isolates from Pampa del Indio herein genotyped cannot be attributed to the PCR protocol for DTU identification. However, DTU selection in mixed infections cannot be excluded. Neither TcI nor TcIII were found among the human parasite isolates despite these DTUs infect humans: TcIII was found in Brazil and Paraguay (F. Del Puerto et al., 2010; Martins et al., 2015) whereas TcI has been found throughout the Americas (Ramírez and Hernández, 2018). Co-infections of TcV and/or TcVI with TcI and TcIII have been revealed in the Argentinean Chaco when using a highly sensitive method directly to blood samples (Monje-Rumi et al., 2015).

To date in Pampa del Indio, we have not found triatomines infected with TcIII using direct typing from feces, and only peridomestic *T. sordida* was found to be infected with TcI (Macchiaverna et al., 2015; Maffey et al., 2012, Supplementary Table S2). Only a few dogs and cats were infected with TcIII and TcI in Pampa del Indio, the majority being infected with TcV or TcVI (Enriquez et al., , 2014, 2013, Supplementary Table S2). Interestingly, TcI-infected dogs and cats were not infectious to xenodiagnosis bugs despite having high parasite loads measured by qPCR from blood samples (Enriquez et al., 2014). Armadillos were found infected with TcIII and *Didelphis* opossums with TcI, both reservoir hosts were highly infective to xenodiagnosis triatomines (Orozco et al., 2013, Supplementary Table S2). Additionally, no host or triatomine has been found infected with TcII in Pampa del Indio so far.

Our findings point to a consolidated domestic transmission cycle of *T. cruzi* in Pampa del Indio, which includes humans, dogs, cats, *T. infestans*, and the predominant TcV and TcVI. Two sylvatic transmission cycles are apparent: one arboreal, involving opossums and TcI, and another

one terrestrial, including armadillos and TcIII. No sylvatic triatomine species has been found infected with *T. cruzi* so far. Peridomestic habitats provide an interface where these transmission cycles eventually meet, and may involve dogs, cats and *T. sordida*, perhaps synanthropic rodents, not humans. Future tasks will seek to identify DTUs from triatomines and humans residing at the same household where dogs and cats were infected with TcI and TcIII to have a more detailed understanding of domestic transmission cycles in this region.

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