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Predominance of hybrid discrete typing units of *Trypanosoma cruzi* in domestic *Triatoma infestans* from the Bolivian Gran Chaco region

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ABSTRACT

In the Gran Chaco region the reinfestation by *Triatoma infestans* remains a major problem for control of Chagas disease. *Trypanosoma cruzi* the agent of the illness presents a broad genetic intraspecific variability which is poorly documented in the Bolivian Gran Chaco. This work presents the identification of the discrete typing units (DTUs) currently recognized for *T. cruzi* in *T. infestans* populations collected before and after residual insecticide spraying in four villages in this region. Before spraying, of 84 samples, the frequencies of the DTUs identified by using the multiplex PCR based on the non transcribed spacer of the mini-exon gene (MMPCR) were 0.21 for TcI, 0.70 for TcII/TcV/TcVI, and 0.17 for TcIII/TcIV and no significant difference was observed after spraying (76 samples). Moreover 13% of the total sample corresponds to *T. infestans* specimens with mixed infection of DTUs of which three were TcII/TcV/TcVI with TcIII/TcIV. The partial sequences of *T. cruzi Gpi* gene obtained from 14 PCR products agree the MMPCR DTU identification and allowed to precise the occurrence of TcIII, TcII and hybrid TcV/TcVI stocks which were not discriminated by the MMPCR.

Given the high prevalence of hybrid stocks, the authors ask whether the recombination event at the origin of hybrids would have taken place in the Gran Chaco where the putative parents are also present.

1. Introduction

Chagas disease, caused by infection with *Trypanosoma cruzi*, is one of the WHO's neglected tropical diseases and is one of the highest disease burdens in Latin American with 8–9 million people infected and 25–90 million at risk (Hotez et al., 2008). In Bolivia, the area of transmission risk covers 80% of the territory and the vectorial transmission is mainly due to *Triatoma infestans* (hemiptera, reduvidae), which has a high rate of infection, nearly 30% (Moncayo and Silveira, 2009). The Gran Chaco region, which spreads over Argentina, Bolivia and Paraguay, is regarded as a hyperendemic area for Chagas disease; this region suffers from serious problems of reinfestation by *T. infestans* despite the actions of vector control by insecticide spraying, and this region is a great challenge for disease control (Cecere et al., 2004; Marcet et al., 2008; Quisberth et al., 2011).

T. cruzi is a pathogenic microorganism that presents broad intraspecific genetic diversity, and genetic characterization is indispensable to answer the question of who transmits the disease because various studies have shown that the genetic diversity of this parasite has a profound impact on its epidemiological, biological and medical characteristics (Tibayrenc et al., 2010). Population genetics based on numerous molecular markers has recognized that clonality is the principal mode of propagation of natural populations of *T. cruzi* (Tibayrenc et al., 1990), and a consensus nomenclature has divided *T. cruzi* into six discrete typing units (DTUs) named TcI to TcVI (Zingales et al., 2009, 2012). At present, this classification seems to be the most parsimonious and useful in epidemiology (Yeo et al., 2005). TcI has the widest distribution, ranging from the southern USA to Northern Argentina and Chile. This DTU is the most frequently sampled in sylvatic cycles, but it is also frequent in domestic cycles and it is the dominant DTU responsible for Chagas disease transmission in the endemic countries that lie to the north of the Amazon basin. Studies show that TcII, V and VI are more likely to be associated with domestic cycles and patients with chronic Chagas disease; TcIII and IV are mostly sampled in tropical forest in sylvatic cycles. However, different DTUs can

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coexist in the same area and within a single host and vector (Brenière et al., 1998; Devillers et al., 2008). In Bolivia, two main groups of *T. cruzi* clones belonging to TcI and TcV, respectively, were identified in the domestic cycle and they have specific epidemiological and clinical properties (Brenière et al., 1998, 2002; Flores-Chavez et al., 2006). To our knowledge, only one study has focused on the characterization of *T. cruzi* infecting *T. infestans* collected in dwellings in the Bolivian Gran Chaco, and both TcI and TcV DTUS were present with a predominance of TcV over TcI (Brenière et al., 1995).

A multiplex PCR based on the nontranscribed spacer of the mini-exon gene (MMPCR) was developed (Fernandes et al., 2001) to discriminate TcI, TcII and TcIII/TcIV. Recently, a strict evaluation of MMPCR among a large sample of *T. cruzi* strains belonging to TcI to TcIV (previously characterized by multilocus typing) showed three DTU tags: a 200-bp PCR product for TcI, a 250-bp product for TcII, V and VI, and a 150-bp product for TcIII and IV (Aliaga et al., 2011). Also, the MMPCR is a rapid typing method (MMPCR) that has already been applied to direct identification of DTUs in feces and stomach contents of triatomines (Bosseno et al., 2006, 2009; Brenière et al., 2007).

Other useful genetic markers to discriminate DTUs are the gene sequence polymorphism (Oury et al., 2005; Yeo et al., 2011; Lauthier et al., 2012) and most particularly the *Gpi* gene (glucose phosphate isomerase), whose sequence variability was previously analyzed in large samples of reference strains belonging to the different DTUs (Broutin et al., 2006; Lewis et al., 2011).

In the present work, the diversity of the *T. cruzi* DTUs infecting *T. infestans* collected in four villages in the Bolivian Gran Chaco before and after spraying was analyzed. The rapid typing method (MMPCR) was applied to a large sample of stomach contents of *T. infestans* and was followed by the partial sequencing of the *Gpi* gene for a sub-sample of DNA extracts, in order to perform a preliminary deeper exploration of the genetic variability.

2. Materials and methods

2.1. Villages and samples studied

The four villages studied are located along the Parapetí River, 300 km southeast of the city of Santa Cruz in the Izozog region. This region belongs to the Boreal Chaco, it has an average altitude of 300 m and the average temperature is 26 °C. It is the driest part of the Chaco, with annual rainfall of about 550 mm from December to April. The inhabitants belong to the Tupi-Guarani indigenous people, although creoles live in some villages. The population density is less than one person per km^2. Among the four villages studied, Tamachindi (19°28′41.21″S; 62°33′49.46″W; 568 inhab.), Rancho Nuevo (19°26′21.59″S; 62°34′04.60″W; 669 inhab.), and San Silvestre (19°21′20.86″S; 62°34′10.19″W; 78 inhab.) are located near each other, while Kuarirenda (19°10′37.88″S; 62°31′36.17″W; 778 inhab.) is located 25 km to the north. Triatomines were collected manually between September and November 2007 by active research of triatomines during the day, indoors and outdoors (in peridomestic structures) without insect repellent (before spraying). During November 2007, all the dwellings in the four villages (100, 128, 23 and 113 dwellings in Tamachindi, Rancho Nuevo, San Silvestre and Kurirenda respectively) were sprayed with alpha-cypermethrin according to the standard methodology used by the National Control Program (50 mg a.i./m^2). Subsequently, similar entomological assessments were carried out in the four villages every 4 months (April, August, December 2008; April, August 2009). During these campaigns (after spraying), new insecticide spraying was only applied to dwellings with triatomines. Before spraying, *T. infestans* specimens for DTU identification were chosen from the positive dwellings taking into account their spatial distribution over the villages to test bugs from different parts of the villages (dwelling infestation rates were 81%, 69.5%, 56.5% and 81.4% in Tamachindi, Rancho Nuevo, San Silvestre and Kuarirenda respectively); adults were generally selected because the stomach contents are more abundant than in nymphs (94.8% of adults of both sexes and 5.2% of 5th nymphal instars). After spraying, the dwelling infestation rates ranged between 10.4–14.8% on April 2008 and 13.2–14.8% on August 2009. The sample included most of the collected adult bugs and some nymphs at the different periods and was composed of 85.4% of adults of both sexes and 14.6% of 2nd, 3rd, 4th, and 5th nymphal instars.

2.2. Extraction of DNA from feces and stomach contents

DNA was extracted from feces of triatomines using DNAzol Reagent (Life Technologies, Villebon-sur-Yvette, France). Briefly, after centrifugation of the feces sample, the supernatant was mixed with DNAzol (1V/7V), centrifuged, and the resulting supernatant precipitated with ethanol for DNA precipitation. DNA was extracted from the stomach contents with the QIAamp DNA mini kit (Quiagen, Courtaboeuf, France), according to the protocol for blood samples with minor modifications (Buitrago et al., 2012). Briefly, a maximum volume of 200 μl of stomach blood was processed and samples less than 200 μl were diluted in PBS to a final volume of 200 μl. In the final step, DNAs were eluted with distilled water in a final volume of 50 or 30 μl for a stomach sample volume less than 20 μl. DNA samples were stored at −20 °C until PCR amplification.

2.3. PCR detection and characterization of T. cruzi DTUs

Part of the total sample before spraying was submitted to PCR amplification using TCZ1 (5′CGAGCTCTTGCCCACACGGGTGCT3′) and TCZ2 (5′CCTCCAAGCAGCGGATAGTTCAGG3′) primers and PCR conditions according to previous description (Moser et al., 1989). These primers were designed to anneal sites at the extremities of the *T. cruzi* satellite DNA repeated unit and detected with high sensitivity all the *T. cruzi* DTUs (Virreira et al., 2003).

PCR amplification of intergenic region of the mini-exon gene was achieved for all the samples with the set of primers and PCR conditions previously described, with slight modifications (Fernandes et al., 2001). DNA was amplified in a 25-μl reaction volume containing 1X reaction buffer, 1.5 mM $MgCl^2$, 50 μM of each nucleotide, 0.2 μM of each primer, 0.5 UI of *Taq* polymerase (Roche Applied Science, Penzberg, Germany). The amplifications were performed in a thermocycler (Eppendorf, Hamburg, Germany), in the conditions already described (Fernandes et al., 2001). PCR products were separated on 3% agarose gel using the molecular weight marker Smart Ladder (Eurogentec, Angers, France) and visualized under UV with Ez-vision (Amresco, Solon, OH, USA).

2.4. Partial sequencing of the glucose-6-phosphate isomerase (Gpi) gene

A 655-bp fragment was amplified with a set of primers, forward (*Gpi*-L) starting at position 591 of the gene (5′-CGCCATGTTGTGAATATTGG-3′) and reverse (*Gpi*-R) 5′-TTCCATTGCTTTCCATGTCA-3′ starting at position 1246, from the DNA sample. DNA was amplified in a 25-μl reaction volume containing 0.75 mM $MgCl_2$, 0.2 mM of each nucleotide, 0.4 μM of each primer, 2.5 UI of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA), and 5 μl of DNA template. The amplification was performed in a thermocycler (BioRad, Marnes-la-Coquette, France), with the following cycle conditions: 94 °C for 3 min; 94 °C for 1 min, 58 °C for 1 min, 72 °C