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Multilocus PCR-RFLP profiling in *Trypanosoma cruzi* I highlights an intraspecific genetic variation pattern

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ABSTRACT

Chagas disease represents a serious problem in public health. This zoonotic pathology is caused by the kinetoplastid *Trypanosoma cruzi* which displays a high genetic diversity falling into six Discrete Typing Units (TcI–TcVI). In Colombia, the prevalent DTU is TcI with findings of TcII, TcIII and TcIV in low proportions. The aim of this work was to observe the genetic variability within TcI using a multilocus PCR-RFLP strategy. We analyzed 70 single-celled clones from triatomines, reservoirs and humans that were amplified and restricted via ten PCR-RFLPs targets across TcI genome, the restriction fragments were used to construct phylograms according to calculated genetic distances. We obtained five polymorphic targets (1f8, HSP60, HSP70, SAPA and H1) and the consensus tree constructed according to these regions allowed us to observe two well-defined groups with close association to the transmission cycles (domestic/peridomestic and sylvatic) of Chagas disease in Colombia. Our findings allowed us to corroborate the previous reported genotypes based on the intergenic region of mini-exon gene. More studies examining the genetic diversity among *T. cruzi* I populations must be conducted in order to obtain a better understanding in regions where this DTU is endemic.

1. Introduction

Chagas disease or American trypanosomiasis is an endemic chronic parasitic and systemic pathology from America that is caused by the kinetoplastid parasite *Trypanosoma cruzi*. Approximately 15 million people are affected by Chagas disease, and 21 million people are at risk of acquiring the infection. In America, the incidence of Chagas disease is approximately 41,200 new cases each year, and causes an average of 12,500 deaths annually (WHO, 2007). The etiological agent, *T. cruzi*, contains important genetic variability that is evident in distinct genotypes or Discrete Typing Units (DTUs) (Brisse et al., 2000; Zingales, 2012). Currently, six DTUs (TcI–TcVI) have been described that are distributed across the American continent, and some genotypes present specific geographical distributions (Lewis et al., 2010; Miles et al., 2009). Within the *T. cruzi* taxon, several hybrids can be found that are likely the result of a natural recombination event and have been corroborated *in vitro* (de Freitas et al., 2006; Gaunt et al., 2003; Westenberger et al., 2005). The TcIII–TcVI hybrid *T. cruzi* groups are a good example of this phenomenon. TcI and TcII are the parental groups, and recombination events between TcI and TcII generated TcIII–TcIV (Zingales et al., 2012). Furthermore, genetic exchange between TcII and TcIII led to the emergence of TcV and TcVI (Lewis et al., 2011).

In Colombia, the DTUs that have been described in humans, insect vectors and host reservoirs are mainly TcI and TcII (Mejía-Jaramillo et al., 2009; Ramírez et al., 2010; Zafra et al., 2008). Several studies using different molecular markers have demonstrated that a high amount of genetic diversity is found in TcI (Cura et al., 2010; Falla et al., 2009; Guhl and Ramírez, 2011; Herrera et al., 2007; Llewellyn et al., 2009a; Ocaña-Mayorga et al., 2010; Ramírez et al., 2011). Analysis of the intergenic region of the mini-exon gene (SL-IR) has revealed five genotypes distributed across the Americas. (Cura et al., 2010; Herrera et al., 2007). Likewise, experiments using microsatellite markers have highlighted two distinct genotypes (Llewellyn et al., 2009a; Ocaña-Mayorga et al., 2010). Moreover, using the coding region of the cytochrome b gene, subgroups clustering in Chile and Colombia have been described (Spotorno et al., 2008; Ramírez et al., 2011). Most of the markers analyzed are non-coding regions of the parasite. Therefore, these analyses have been based on possibly neutral variation. The implications of the genetic variability within TcI recently garnered significant attention because it has been associated with the clinical manifestations and the development of cardiomyopathies in Argentina and Colombia (Burgos et al., 2010; Ramírez et al., 2010; Zafra et al., 2011). Additionally, there are differences between the humoral

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responses of patients with chronic Chagas disease (Santos et al., 2009; Macedo and Segatto, 2010; Ramírez et al., 2009).

Restriction pattern analysis of the coding regions using PCR-RFLPs has been performed to confirm the high degree of polymorphism within the parasite (Rozas et al., 2007; Sturm et al., 2003) and to develop algorithms to accurately assess the DTU of *T. cruzi* isolates. (Lewis et al., 2009; Rozas et al., 2008). These types of analyses have always been performed with a restricted number of TcI isolates, and the same isolates have been used in all of the published studies. The objective of this study was to evaluate the genetic variability within TcI by performing a multilocus PCR-RFLP analysis using the coding regions of a set of well-characterized TcI clones that were isolated from humans, insect vectors and reservoirs from different geographical regions in Colombia.

2. Materials and methods

2.1. Study isolates

Seventy clones were selected from *T. cruzi* isolates from different hosts and geographical regions in Colombia (Table 1). These isolates included one reference strain from *T. c. marinkellei* that was used as an out-group. The isolates were cloned using limiting dilution. Each clone was harvested in LIT-biphasic media until the exponential phase of growth. Aliquots of 200 μL were used to perform DNA extraction using the Qiagen Mini kit Qiamp according to the manufacturer's instructions. Each clone was typed using SL-IR with the primers TCC, TCI and TCII in a multiplex assay (Souto et al., 1996) and by amplifying the divergent domain D7 from the 24Sα rDNA subunit (Brisse et al., 2000).

2.2. Selection of loci and restriction enzymes

Nine coding regions, one non-coding region, a control region and the SL-IR, were selected for analysis. Because of no DNA sequence information exists for the primary restriction targets of TcI, all nine regions were selected from the studies by Rozas et al., 2008; Lewis et al., 2009; Van der Auwera et al., 2010, which reported divergent branches of the TcI clades in the phylograms. The following regions were selected for analysis: the 72 kDa glycoprotein (Gp72), Histone 1 (H1), calcium binding protein (1f8), Heat Shock Protein 60 (HSP60), the 63 kDa glycoprotein (Gp63), Heat Schock Protein 70 (HSP70), Surface Anchored Protein Antigen (SAPA), Histone 3 (H3) and Glucose Phosphate Isomerase (GPI) (Rozas et al., 2008; Van der Auwera et al., 2010). The non-coding region selected was the Internal Transcribed Spacer (ITS). Because of recent reports of variability within TcI isolates (Cuervo et al., 2002; Luna-Marín et al., 2009) (Table 2), three different enzymes were selected for each region reported in the literature. For the control region, the SL-IR was developed using an in silico search in Webcutter 2.0 and the TcI sequences from GenBank were employed to select the enzymes that then allowed us to discriminate among the previously described genotypes within TcI.

2.3. Amplification and restriction analysis

From the selected regions we used specific primers to PCR amplify each region. A PCR master mix containing 10X GoTaq Hotstart Green (Promega), 10 μM of each primer, three microliters (50 ng/μL) of DNA and water up to a final volume of 25 μL. The same reaction mix was used to amplify all of the genes except Gp72 and ITS, which also contained 50 mM $MgCl_2$. A BIORAD thermocycler and thermal profiles were used as described elsewhere (Lewis et al., 2009; Rozas et al., 2008; Van der Auwera et al., 2010). For the restriction analysis, we used 20 μL of the amplification product from each region and performed the restriction assays according to manufacture's recommendations (FERMENTAS) (Table 2). The restriction products were then analyzed using electrophoresis on a small fragment agarose gel stained with ethidium bromide that was run at 2.5–3.0 V/cm for at least 5 h. Each gel was digitalized using the BIORAD chemidoc system. For the accurate determination of band fragment sizes in the SL-IR region, the digestion products were submitted to fragment size determination using a microchip (2100 Bioanalyzer capillary electrophoresis system; Agilent Technologies, Karlsruhe, Germany) with 5 μL of digestion avoiding the need of sequencing the PCR products.

2.4. Phenetic analyses

The results of the restriction assays from the PCR-RFLPs were phenetically analyzed to determine the genetic distances between the evaluated clones. Thus, an array of characters and a matrix of categorical variables were created; a value of 1 indicated the presence of a band, and the value 0 indicated the lack of a band. The intensity of the band was not considered, and the construction of matrices was performed by three different operators to avoid operator-dependent bias (type I error). A consensus matrix was created for each gene, and each matrix was concordant between the three operators. The matrixes were analyzed using PHYLIP v.3. and the package RESTDIST (Restriction Fragment Distance, Modification of Nei and Li restriction fragments distance method) (Li, 1981). The distance matrix of each gene was used to construct phenetic trees using the algorithm UPGMA (Unweighted Pair Group Method with Arithmetic averaging). Likewise, to test the robustness of the nodes, the resulting tree was evaluated using SEQBOOT (Bootstrap Analysis) with 1000 iterations. The phenetic trees were visualized using Figtree v 3.0. Eleven different trees were obtained, and the categorical data from all of the evaluated regions were used to construct a consensus phenetic tree.

3. Results

3.1. Restriction analyses

The selected regions were analyzed with three different restriction enzymes. Only five of the ten regions demonstrated polymorphic restriction patterns with the following enzymes: 1f8 (Alw21I), H1 (AatII), HSP60 (EcoRV), HSP70 (Csp6I and BseXI) and SAPA (HpaII). Similarly, the use of the restriction enzymes BstUI and RsaI permitted the discrimination of three distinct TcI genotypes based on the SL-IR region. When the restriction was performed with BstUI for the TcIa genotype, three fragments of 53, 109 and 151 bp were obtained. For the TcIb genotype, four fragments of 32, 54, 77 and 151 bp were obtained. For the TcId genotype four fragments of 31, 54, 77 and 143 bp were obtained. The restriction pattern generated by RsaI for the TcIa genotype was indicated by a single band of 350 bp, the TcIb genotype was indicated by two bands of 81 and 233 bp, and the TcId genotype was indicated by two bands that were 80 and 225 bp. These assays accurately discriminated between the previously identified SL-IR TcI genotypes using the BstUI enzyme. The clone from isolate X380, which was previously characterized as TcIc, clustered with TcId when the restriction analyses were performed with BstUI.

3.2. Phenetic analyses

The phenetic trees were constructed using the results from the restriction analyses and the calculated genetic distances (Fig. 1). Five regions were polymorphic, and region 1f8 had a high amount of genetic diversity among the clones analyzed (Fig. 1A). The HSP60