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# Oral transmission of Chagas disease: Typing of *Trypanosoma cruzi* from five outbreaks occurred in Venezuela shows multiclonal and common infections in patients, vectors and reservoirs

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In Venezuela six episodes of oral transmission of Chagas disease (OChD) have been described, being the one reported in 2007 with a total of 103 people infected the largest worldwide. This work shows the use of three molecular markers (mini-exon gene and domains 24Sα and 18S of the ribosomal RNA) to characterize the infecting *Trypanosoma cruzi* strain of patients, reservoirs and vectors involved in five of the six OChD outbreaks. For this, 28 *T. cruzi* isolates were characterized by PCR, and the products of these reactions cloned and sequenced to reveal the existence of different TcI SL-IR genotypes. We also describe a new PCR assay able to discriminate between TcIb and TcId parasite populations. In summary, we have identified mostly parasites with the TcId haplotype and multiclonal populations with predominance of haplotype TcId (65.2%). Additionally, populations of haplotypes TcIb, TcIa and mixtures (TcId + TcIb, TcId + TcIa, TcIb + TcIa) are recurrent in samples obtained from children. The analysis of the SL-IR motif showed two clones depicting a different motif that could be an evidence for a possible hybrid haplotype between TcIa and TcIb (haplotype TcIa/Ib). Interestingly, in a single patient haplotype differences between *T.cruzi* isolates obtained pre and post-treatment were found. In conclusion, our findings show that in order to understand the pathogenic mechanisms involved in the orally adquired Chagas disease there is a need to join efforts to study *T. cruzi* haplotypes, their tissue tropisms and their susceptibility to chemoteaphy.

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## 1. Introduction

Chagas disease (ChD) which is included by the World Health Organization (WHO) among the group of "neglected or forgotten diseases" is endemic in America. An estimated 15–16 million people are infected and 75–90 millions are exposed to the infection (Moncayo and Silveira, 2009). Moreover, being a zoonosis, involving domestic and wild reservoirs, the control of the transmission results very difficult. Additionally, there are limitations on the availability of efficient drugs, and deficiencies in the manufacture and distribution of the two available medicaments namely nifurtimox and benznidazol. The recent trends on global migrations from rural to urban areas, and from endemic to non-endemic countries, increase the threat of infection by ChD (Franco-Paredes et al., 2007; Rassi et al., 2010).

Although ChD is mostly transmitted by direct exposure to infected triatomine feces, there are other forms of transmission such as blood transfusion, congenitally and oral infections which are becoming more recurrent in non-endemic areas (Rassi et al., 2010).

Outbreaks of oral Chagas Diseases (OChD) have been reported in Brazil, Colombia, Bolivia and Venezuela (Alarcón de Noya et al., 2010b) with several acute cases infected by eating food contaminated with vector feces, or less likely by the spray of marsupials anal secretions (Dias and Coura, 1997). The largest worldwide outbreak of OChD occurred in Venezuela in 2007, with a total of 103 infected people (Alarcón de Noya et al., 2010a).

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The biological, biochemical and genetic diversity of *Trypanosoma cruzi* strains has long been recognized and may explain the variability observed in its eco-epidemiological, pathological, clinical, virulence and response to treatment (Macedo and Pena, 1998; Campbell et al., 2003; Miles et al., 2009). Over the years, numerous approaches have been used to characterize the structure *T. cruzi* population aiming to define the number of relevant subgroups.

At a satellite symposium held in Buzios, Brazil in 2009 a new nomenclature to designate distinct Taxonomic Units (DTUs) was established namely: TcI, TcII (formerly TcIIb), TcIII (formerly TcIIc), TcIV (formerly TcIIa), TcV (formerly TcIId) and TcVI (formerlyTcIIe) (Zingales et al., 2009). This classification was based on polymorphisms found in the mini-exon gene's intergenic spacer region (Fernandes et al., 1998). The mini-exon gene is involved in the post-transcriptional events during mRNA processing, and it has been proposed as an important molecular marker given its essential role as a control mechanism for differential protein expression and its high variability within *T. cruzi* populations (Thomas et al., 2005). Herrera et al. (2007) based on single nucleotide polymorphisms (SNPs) and insertions/deletions at the intergenic spacers of the multicopy spliced-leader gene (SL-IR), proposed four TcI haplotypes in Colombia. Considering the distinctive patterns among the cycles of transmission of ChD (vectors, reservoirs and human) these haplotypes were recently designated as Ia–Id by Falla et al. (2009).

In this paper we used of the above mentioned molecular biomarkers to characterize the infecting *T. cruzi* strains isolated from patients who acquired OChD in five of the six outbreaks that occurred in Venezuela. Moreover, a comparison of the pattern of infecting strains in adult and children at the acute phase of the sickness, and six months/one year post treatment administration with Nifurtimox or Benznidazole suggests a possible influence of the treatment on the type of infecting parasite. Finally, we designed a pair of primers specific of TcIb that allowed us to use a PCR for an easy identification in single and multiclonal infections by this haplotype.

## 2. Materials and methods

### 2.1. Parasite culture

Parasites were cultured in LIT medium (supplemented with 5% bovine fetal serum) and sub-cultured every 15 days during the exponential growth phase.

### 2.2. DNA extraction and quantitative analysis

Parasites were collected at logarithmic phase and centrifuged at 3000 rpm. The QIAamp® DNA blood midi kit (QIAGEN) was used for DNA extraction following the manufacturer's recommendations. The genomic DNA was then preserved at 4 °C until use. DNA purity and concentration was determined by 0.8% agarose gel electrophoresis and in a Nanodrop ND-1000 (NanoDrop Technologies, Houston, TX, USA) apparatus at 260/280 nm wavelengths.

### 2.3. Molecular typing of T. cruzi isolates

Each one of the *T. cruzi* isolates was characterized by PCR (in a GeneAmp PCR System 2700 Thermal Cycler) using three molecular targets/markers: first PCR was carried out to amplify the intergenic region of the mini-exon gene, followed by amplification of the domains of the 24S and 18S ribosomal RNA. The expected amplicon sizes for each marker and each DTU are shown in Fig. 1A.

The PCR amplification for the intergenic region of the mini-exon gene was carried out in 25 μl of reaction mixture containing: 100 ng/μL DNA, 1.5 mM MgCl2, 25 mM of dNTPs, 0.2 μM of each primer described by Souto et al. (1996) (TC, *TCI* and TC2) and 0.5 U/μL of Taq DNA polymerase (Biotools B&M labs). The amplification consisted of a 94 °C denaturing step for 1 min, followed by 27 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 5 min. The expected amplified products had 300 bp for TcII, III, IV, V or VI, and 350 bp for TcI. These products were separated by electrophoresis in 1.6% agarose gel and visualized after ethidium bromide staining.

The amplified products were excised from the gels and purified using a QIAEX® II gel extraction kit (QIAGEN) and subsequently cloned into a pGEM-T easy Vector (Promega, USA). To detect the different TcI SL-IR genotypes we sequenced both strands of at least three clones per sample using the Sanger method in a 3700 Applied Biosystem DNA automatic sequencer.

The D7 divergent domain of the 24Sα rRNA was amplified following the methodology proposed by Souto et al. (1996), using the D71 and D72 primers with 30 cycles of amplification (1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C) followed by an elongation step of 5 min at 72 °C.

The PCR amplification of the size-variable domain of the 18S rRNA sequence was carried out as described by Clark and Pung (1994), using the V1 and V2 primers and 30 cycles (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) followed by a final elongation of 5 min at 72 °C. All amplification cycles were performed in a thermocycler GeneAmp PCR System 2700.

### 2.4. Sequence analysis of the SL-IR microsatellite motif

Electropherograms of forward and reverse sequences were edited using the Sequence Scanner software V 1.0 (Applied Biosystems, 2005). Sequences were aligned using the ClustalW algorithm (Thompson et al. 1994) and manually adjusted. Reference genotypes for each haplotype were obtained from the GenBank database: CGC (AM259467), FChC (AM259469) and PALC (AM259473).

### 2.5. NoGap-3′ specific PCR assay

Based on the SL-IR sequence from parasites belonging to haplotype TcIb, a specific primer, named noGAP-3′ was designed which together with TC primer allows discriminating TcIb populations. PCR reactions were carried out in a final volume of 100 μL of reaction mix containing 1X commercial buffer, 1.5 mM $MgCl_2$, 25 mM of dNTPs, 0.2 mM of each primer and 0.5 U/μL of Taq Polymerase (Biotools B&M labs). The PCR was carried out with the same program used for SL-IR amplification. Products were analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining and UV visualization.

## 3. Results

### 3.1. Patient and sample selection

Genomic DNA (gDNA) was purified from parasites isolated by the hemoculture of total peripheral blood drawn from patients infected in the OChD outbreaks that occurred in the Capital District, Vargas and Táchira states. This DNA was used as template for the PCR amplification of the intergenic region of the mini-exon gene. First, samples from ten adults and seven children who still had not received the treatment were selected. Additionally, six samples from treated patients (one adult and two children treated with Nifurtimox and three children treated with Benznidazole) were also

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