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Research paper

Simple methodology to directly genotype *Trypanosoma cruzi* discrete typing units in single and mixed infections from human blood samples

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ABSTRACT

Different DNA markers to genotype *Trypanosoma cruzi* are now available. However, due to the low quantity of parasites present in biological samples, DNA markers with high copy number like kinetoplast minicircles are needed. The aim of this study was to complete a DNA assay called minicircle lineage specific-PCR (MLS-PCR) previously developed to genotype the *T. cruzi* DTUs TcV and TcVI, in order to genotype DTUs TcI and TcII and to improve TcVI detection. We screened kinetoplast minicircle hypervariable sequences from cloned PCR products from reference strains belonging to the mentioned DTUs using specific kDNA probes. With the four highly specific sequences selected, we designed primers to be used in the MLS-PCR to directly genotype *T. cruzi* from biological samples. High specificity and sensitivity were obtained when we evaluated the new approach for TcI, TcII, TcV and TcVI genotyping in twenty two *T. cruzi* reference strains. Afterward, we compared it with hybridization tests using specific kDNA probes in 32 blood samples from chronic chagasic patients from North Eastern Argentina. With both tests we were able to genotype 94% of the samples and the concordance between them was very good (kappa = 0.855). The most frequent *T. cruzi* DTUs detected were TcV and TcVI, followed by TcII and much lower TcI. A unique *T. cruzi* DTU was detected in 18 samples meantime more than one in the remaining; being TcV and TcVI the most frequent association. A high percentage of mixed detections were obtained with both assays and its impact was discussed.

1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, affect at least eight million people (WHO, 2014), and despite recent advances in disrupting vector transmission, it remains a major public health problem in Latin America (Rassi et al., 2010; Schofield et al., 2006). Furthermore, human migrations increase the disease burden in non-endemic areas such as the United States, Canada, Japan, Australia and many European countries (Schmunis and Yadon, 2010).

Chronic human infection can progress to cardiac and/or digestive disease with possible subsequent death, with approximately 14,000 deaths annually (WHO, 2007). The variability in the symptoms of Chagas disease may be correlated with specific genetic markers in the parasite or in the host, although it is likely that both would affect the outcome of the infection (Sturm et al., 2003). *T. cruzi* is genetically classified into six intra-species lineages, currently called discrete typing units (DTUs): TcI–VI (Zingales et al., 2012). This intraspecific diversity has been demonstrated by differences in morphology of blood forms, virulence, pathogenicity, susceptibility to chemotherapeutic agents, immunological properties and infectivity in host cells (Murta and Romanha, 1999). Moreover, it has also been associated with geographical distribution (Miles et al., 2009). While TcI is mainly found in Central America and as far north as the USA; TcII, TcV and TcVI predominately in the southern cone countries. Furthermore human TcII infection has also been reported in Colombia (Zafra et al., 2008). The different genotypes may also be associated with human and natural sylvatiac and domestic transmission cycles (Anez et al., 2004). At first, former studies have been associated *T. cruzi* TcI with the sylvatic cycle, and the others DTUs with the domestic cycle. However, this epidemiological association is not already correct, because all DTUs have been isolated in both sylvatic and domestic cycles as well (Yeo et al., 2007). Although TcIII, and TcIV are predominantly found in sylvatic transmission cycles, TcIII was recently found in human infections (Martins et al., 2015) and occasionally isolated from domestic dogs (Zingales et al., 2009); while TcIV was also isolated from humans, mainly infected by oral transmission (Carrasco et al.,

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2012; Monteiro et al., 2012). Classification of *T. cruzi* was based on a variety of molecular markers including polymorphism of rDNA and miniexon (Murthy et al., 1992; Souto and Zingales, 1993), RFLP (Bastrenta et al., 1999; Morel et al., 1980), random amplified polymorphic DNA (RAPD) (Steindel et al., 1993), multilocus enzyme electrophoresis (MLEE) (Tibayrenc and Ayala, 1987), and hybridization with kinetoplast (kDNA) probes (Breniere et al., 1992; Solari et al., 1991). However, the most widely used methods for differentiating among *T. cruzi* DTUs are based on nuclear markers (Burgos et al., 2007; Cura et al., 2015; Freitas et al., 2005; Lewis et al., 2009; Rozas et al., 2007), although with low sensitivity, especially when genotyping was performed on human samples from chronic infections. Indeed, some studies have shown that a single molecular marker has insufficient resolution for classifying all *T. cruzi* isolates (Brisse et al., 2001; Burgos et al., 2007).

kDNA, instead, has multiple minicircles copies, with the advantage of perform a direct parasite detection from biological samples by amplification of hypervariable regions on the minicircles (mHVR). Beyond kDNA is not currently used as reference target for *T. cruzi* genotyping, however, high sensitivity and reproducibility has been proven with this marker, when DNA concentrations are optimized to avoid unspecific reactions (Breniere et al., 1991; Rodriguez et al., 2009) (del Puerto et al., 2010). Taken into account this fact, we have previously developed a nested PCR to amplify the major minicircle sequences of DTUs TcV and TcVI. With this method, named MLS-PCR (Minicircle Lineage Specific PCR), we have achieved high specificity for DTU detection and high sensitivity for DTU TcV, been lower the sensitivity for TcVI detection (Diez et al., 2010).

In this paper we extend the scope of this technique for DTUs TcI and TcII detection and to improve the sensitivity for DTU TcVI to identify and diferentiate these genotypes in single and mixed *T. cruzi* infections directly from human samples.

2. Materials and methods

2.1. T. cruzi strains and sample preparation

Reference strains used in this work were: Sylvio X10 cl1, spAI, sp31, sp104 cl1, LQ, and 13,379 cl7 belonging to TcI; Tu18 cl2, IVV cl4, CBB cl3, and vTV belonging to TcII; M 5631 cl5, and P109 cl2, belonging to TcIII; CAN III cl1 belonging to TcIV; JGG, XHCH 56, NR cl3, SC43 cl1 and MN cl2 belonging to TcV; and finally CL Brener, Tul cl2, CHE, and V195 cl1 belonging to Tc VI (Table 1).

All *T. cruzi* strains were cultured in liver infusion tryptose (LIT) at 28 °C as previously described (Velazquez et al., 2008). Breaking down of concatenated DNA and subsequent purification was carried out as described below for human blood samples.

2.2. Patients and blood sample

This study was carried out with chronically infected individuals from Hospital Central de Reconquista, and Centro de Investigaciones sobre Endemias Nacionales, Universidad Nacional del Litoral, Santa Fe province, Argentina, who were positive by conventional serology, ELISA and Indirect hemaglutination. Seven milliliters of blood were mixed with an equal volume of buffer Guanidine HCl/EDTA (6 M/0.2 M) in a polypropylene tube, boiled in a water bath for 15 min to shear and physically decatenate the maximum of minicircle DNA molecules from the kinetoplast network, and kept at 4 °C until use for PCR assays. Afterward, aliquots of 200 μL guanidine/EDTA of lysates were subjected to phenol–chloroform extraction, ethanol precipitation, and DNA resuspension in equal volumen of sterile distilled water. Each set of purifications were performed with the respective positive and negative controls.

2.3. Informed consent

Informed consent was obtained from all human adult participants. The project was approved by the Ethical Committee of the Biochemistry Faculty, Universidad Nacional del Litoral, Argentina.

2.4. Amplification of kDNA minicircle high variable regions (mHVR)

Fragments of 330-bp kinetoplast DNA from *T. cruzi* strains and human blood samples were amplified using primers S121 (5′-AAA TAA TGT ACG GG(T/G) GAG ATG CAT GA-3′) and S122 (5′-GGT TCG ATT GGG GTT GGT GTA ATA TA-3′), as previously described (Velázquez M et al., 2008). These primers were designed from the conserved regions of the minicircles that flank mHVRs (Avila et al., 1991). PCR was carried out in 50 μL of the reaction mixture containing 250 μM of each dNTP, 0.25 μM of each primer (Invitrogen, USA), 3 mM $MgCl_2$, 1.5 IU *Taq* DNA polymerase (Invitrogen, USA), and template DNA. Amplification was performed in a Mastercycle personal Eppendorf thermal cycler programmed for initial denaturation at 94 °C for 3 min followed by 32 cycles of 1 min at 94 °C, 1 min at 59 °C and 1 min at

Table 1
Reference strains representing the six *Trypanosoma cruzi* discrete typing units (DTUs). Host and geographical origin, DTU classification, and methods used for genotyping.

Strain	DTU	Host origin	Geographical origin	Method	Reference
Sp104 cl 1	TcI	*Mepraia spinolai*	Cobarbalá, Chile	optimized MLST	Breniere et al. (1991)
Sylvio X10 cl1	TcI	*Homo sapiens*	Belen, Brazil	MLEE	Miles et al. (1978)
13,379 cl7	TcI	*Homo sapiens*	Bolivia	MLEE	Tibayrenc and Miles (1983)
spAI	TcI	*Mepraia spinolai*	Flor del Valle, Chile	MLEE	Solari et al. (1998)
sp31	TcI	*Mepraia spinolai*	Flor del Valle, Chile	MLEE	Solari et al. (1998)
LQ	TcI	*Homo sapiens*	La Isla, Chile	MLEE	Barnabé et al. (2001)
Tu18 cl2	TcII	*Triatoma infestans*	Iquique, Chile	MLEE, RAPD	Breniere et al. (1998)
CBB cl3	TcII	*Homo sapiens*	IV Region, Chile	MLEE	Breniere et al. (1991)
Ivv cl4	TcII	*Homo sapiens*	Limari, Chile	MLEE, RAPD	Breniere et al. (1998)
vTV	TcII	*Triatoma infestans*	Iquique, Chile	Cyt b sequencing	Arenas et al. (2012)
M 5631 cl5	TcIII	*Dasypus novemcinctus*	Pará, Brazil	MLEE	Miles et al. (1978)
X109/2	TcIII	*Canis familiaris*	Makthlawaiya, Paraguay	Optimized MLST	Chapman et al. (1984)
CAN III cl1	TcIV	*Homo sapiens*	Belem, Brazil	MLEE	Miles et al. (1978)
SC43 cl1	TcV	*Triatoma infestans*	Santa Cruz, Bolivia	MLEE	Tibayrenc and Miles (1983)
MN cl2	TcV	*Homo sapiens*	Ilapel, Chile	MLEE	Brisse et al. (2000)
NR cl3	TcV	*Homo sapiens*	Salvador, Chile	MLEE, RAPD	Brisse et al. (1998)
JGG	TcV	*Homo sapiens*	IV Region, Chile	MLEE	Barnabé et al. (2001)
XhCh 56	TcV	*Homo sapiens*	Chile	MLEE	Barnabé et al. (2001)
Tula cl2	TcVI	*Homo sapiens*	Tulahuen, Chile	MLEE	Tibayrenc and Ayala (1987)
CH2	TcVI	*Triatoma infestans*	San Pedro Atacama, Chile	MLEE	Solari et al. (1998)
Cl Brener	TcVI	*Triatoma infestans*	Rio Grande do Sul, Brazil	MLEE, RAPD	Brisse et al. (1998)
V195	TcVI	*Triatoma infestans*	Eastern Region, Paraguay	Molecular karyotype	Solari et al. (1998)