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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

Iván A. Bontempi [a], María L. Bizai [b], Sylvia Ortiz [c], Silvia Manattini [d], Diana Fabbro [b], Aldo Solari [c], Cristina Diez [e,*]

[a] Laboratorio de Tecnología Inmunológica, Facultad de Bioquímica y Cs Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[b] Centro de Investigaciones sobre Endemias Nacionales, Facultad de Bioquímica y Cs Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[c] Program of Cellular and Molecular Biology, Biomedical Sciences Institute, Faculty of Medicine, University of Chile, Santiago, Chile
[d] Hospital Central de Reconquista, Santa Fe, Argentina
[e] Laboratorio de Biología Molecular e Inmunología Aplicadas, Facultad de Bioquímica y Cs Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina

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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.,

* Corresponding author at: Ciudad Universitaria, Pje El Pozo, cc: 242, Santa Fe, Argentina.
E-mail addresses: iabontempi@gmail.com (I.A. Bontempi), cridiez@gmail.com (C. Diez).

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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)

72 °C, with a 3 min final extension step at 72 °C. One-tenth of the reaction product was electrophoresed on a 1.5% agarose gel, and visualized by staining with ethidium bromide.

2.5. Cloning of mHVR

Amplification products of mHVR from *T. cruzi* strains and clones were purified with the Wizard SV Gel and PCR Clean-Up System kit purification (Promega, USA). DNAs were later cloned using the pGEM® T easy vector (Promega, USA) in the fragment: vector molar ratio indicated by the manufacturer. Then, randomly selected *Escherichia coli* colonies were transferred to a nylon membrane Hybond-N^+ (Amersham, USA) (Sambrook et al., 1989) for hybridization with the DTU-specific DNA probes.

2.6. Hybridization with specific radiaoactive probes

Transferred *E. coli* colonies that contain mHVR, were individually hybridized against a panel of six P^{32} radiolabelled *T. cruzi* genotype-specific probes: sp104 cl1 and spCol 108, belonging to TcI (Arenas et al., 2012; Coronado et al., 2006), CBB cl3 and 36 (Coronado et al., 2006; Gonzalez et al., 2010) belonging to TcII, NR cl3 belonging to TcV, V195 cl1 belonging to TcVI (Coronado et al., 2006); and analyzed in a Personal Molecular Imager-FX (Bio-Rad, USA), as described (Coronado et al., 2006). The probes were constructed as described (Veas et al., 1991).

2.7. Sequence analysis and design of minicircle primers

Plasmid DNA for all selected clones were obtained by the Wizard® Plus SV Minipreps DNA Purification method (Promega, USA), and were sequenced with the SP6 oligonucleotide (automated sequencer AB13730XL, Instituto de Biotecnología INTA, Castelar, Argentina).

The sequences were aligned to each other and analyzed for similarity rate among them and with any previously indexed kDNA sequence of *T. cruzi*, using Bioedit Software and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Specific oligonucleotide primers for each DTU were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).

2.8. Minicircle lineage specific-PCR (MLS-PCR)

T. cruzi genotyping were performed as previously described (Diez et al., 2010), using quantified 330 bp mHVR amplicons as template. Quantification was done using a standard DNA curve of 300 bp DNA fragment from 100 bp ladder (Promega, USA). The intensity of electrophoresis gel images obtained by digital capture was compared with the DNA intensity taken as reference, using the Scion Image analysis software (Scion Corp., Frederick, MD). Dilutions were then performed to obtain standardized mass of DNA template (Velazquez et al., 2008).

To carry out the nested PCR, internal oligonucleotide pairs for each DTU were used. Oligonucleotides sequences can be seen in Table 2, with the corresponding annealing temperature, *T. cruzi* DTU that amplify, and amplicon size. PCR conditions were the same as for mHVR-PCR, only changing annealing temperatures. We repeated these assay three times.

The first PCR as well as the nested-PCR were done in duplicate with the corresponding positive and negative controls. Within the latter, a reaction control (water), a purification control (purified water), and a sample loading control (water), were included. In order to detect any possible contamination in the dilution step of the amplicons, the negative controls products of the first PCR were also included in the nested PCR.

Table 2
Oligonucleotide primers for each MLS-PCR, annealing temperature (Ta), DTU that amplify with their corresponding amplicon sizes.

Primers	DTU	Sequence	Ta (°C)	Product (bp)
X61-f	TCI	5′-TGATATACCACTACCACCGTTACC-3′	65	111
X61-r		5′-AGATAGAGTGCGTGTGAGATGAGT-3′		
X71-f	TCI	5′-ATTTCTTATACCACCATTTTA-3′	47	187
X71-r		5′-TATGGTTGTTTAGTTATTGTAGT-3′		
IB1-f	TCII	5′-ACACGCAAACCACCTCTACTACA-3′	63	168
IB1-r		5′-ATTATGTTATGTGTCTGTGGTGGA-3′		
GrP-f	TCV	5′-GGGATTAGGGTATACTTAGTTGC-3′	55	206
GrP-r		5′-TTCCAACCCCACAAATGATA-3′		
GrO-f	TCV	5′-GGCTTAGGGTGTGGATAGG-3′	55	200
GrO-r		5′-ATCGCGAAACCCATACAA-3′		
Grp1-f	TCVI	5′-ATCCAGACCCCAATTTTACTAC-3′	55	175
Grp1-r		5′-ATGTGATTGGATAGGTGATAGAT-3′		
Tul11-f	TCVI	5′-AATTTCCGTATTTTCTGA-3′	39	163
Tul11-r		5′-TTTATAGTGGTATGTTTGAGTT-3′		

2.9. Statistics

The Cohen's kappa coefficient (kappa) was used to assess the agreement between hybridization tests and MLS-PCR for *T. cruzi* genotyping. The kappa values were interpreted as described by Landis and Koch (1977): 0.00–0.20 = weak agreement; 0.21–0.40 = regular agreement; 0.41–0.60 = moderate agreement; 0.61–0.80 = good agreement; 0.81–1.00 = very good agreement. Negative values were interpreted as equal to 0.00.

3. Results

3.1. Screening and selection of minicircle sequences using DTU-specific probes

To identify the most representative minicircle sequences of each DTU, we amplified mHVR from Sylvio X10 cl1 (DTU TcI), CBB cl3 (DTU TcII), and Tul cl2 (DTU TcVI) strains, and cloned the 330 bp purified products into a prokaryotic system. After checking that colony hybridization signal was not related to the amount of transferred DNA, we picked up one hundred randomly selected clones from each *T. cruzi* DTU, and subjected them to colony hybridization with *T. cruzi* DTU-specific DNA probes. We selected the clones presenting the highest hybridization signal with the corresponding *T. cruzi* DTU probes and with low or no signal with the non-related ones (Table 3).

3.2. Sequence analysis

To analyze the selected mHVR sequences, we have not considered the S121 and S122 oligonucleotide regions and a 50 bp fragment adjacent to S121, which is part of the minicircle conserved region present in all the *T. cruzi* DTUs. When we aligned and compared sequences belonging to the same *T. cruzi* DTU we observed less than 5% identity among all them. On the other hand, when we compared the sequences

Table 3
Sequenced clones, parental strain, and corresponding *T. cruzi* DTU.

DTU	Strain	Clone
TcI	Sylvio X10 cl1	TcI1
		TcI2
		TcI3
		TcI4
TcII	CBB cl3	TcII1
		TcII2
		TcII3
TcVI	Tul cl2	TcVI1
		TcVI2
		TcVI3

from *T. cruzi* DTUs TcII and TcVI, with those indexed in GenBank, we found high identities to sequences belonging to TcII and TcVI, respectively. For example, we found a 94% identity between TcII1 and the awmp248 sequence from Mas cl1 (Telleria et al., 2006); and 99% identity between TcVI1 and the JP701 sequence from X154/7 (Telleria et al., 2006). However, TcI sequences showed low percentage identity with reported sequences. For this DTU, the highest degree of identity (31%) was achieved between Tcl4 and the mp12 sequence from sp104 cl1 (Telleria et al., 2006).

3.3. Minicircle lineage specific-PCR (MLS-PCR)

To complete the method of MLS-PCR for genotyping of the four major DTUs found in human infections, we designed primers for the DTUs TcI, TcII and TcVI. According to the Blast results, we selected the TcII1 and TcVI1 sequences to design the primers IB1 and Tul11, respectively (Table 2). However, given the low identity found within TcI, the design of primers was based on hybridization results. Those minicircle sequences having the highest hybridization signals with the specific DTU probes, and low or no signal with the non-specific ones were chosen (not shown). Thus, we used the Tcl2 and Tcl3 sequences to design the primers X6I and X7I, respectively.

With the designed primers we evaluated the specificity and detection limit of MLS-PCR (nested PCR). We used decreased amounts of mHVR amplicons (first PCR) from one *T. cruzi* strain belonging to a specific DTU and two strains from two different DTUs. Primers Tul11 and IB1 specifically amplified products of 163 and 168 bp from Tul cl2 and CBB cl3 330 bp templates, respectively. The detection limit for these set of primers was 0.3 pg. Both X6I and X7I specifically amplified sp104 cl1, with 111 bp and 187 bp products, respectively. The detection limit for both set of primers was 30 pg. As none of *T. cruzi* strains belonging to the other DTUs was amplified in any of the concentrations tested, we choose a DNA mass of 300 pg to standardize MLS-PCR for these sets of primers. For the nested PCR performed with GrP and Grp1 primers, we used the previously standardized mass of 0.3 pg (Velazquez et al., 2008).

3.4. MLS-PCR in reference strains

After establishing PCR conditions with the designed primers, we evaluated them in twenty two reference *T. cruzi* strains belonging to all *T. cruzi* DTUs (Table 4). Primers X61 and X71 specifically amplified strains Sylvio X10 cl1, sp104 cl1, and 13,379 cl7. Moreover, X61 amplified spAI, but neither X61 nor X71 amplified DNA from the *T. cruzi* strains sp31 or LQ which are variants of TcI as described (Breniere et al., 1991). Meantime primers IB1 specifically amplified strains CBB cl3, Tu18 cl2, and vTV; they did not amplify IVV cl4. Finally, primers Tul11 only amplified Tul cl2. To check whether those specific sequences were present in strains from the other DTUs but to a lesser extent, all assays were repeated without diluting DNA (average amount 90 ng). Results were the same as those observed when using 300 pg confirming primers specificity. We have also evaluated the specificity of the previously reported primers with the twenty two reference strains. That is, primers GrO and GrP for TcV, and Grp1 for TcVI. The former two sets of primers specifically amplified all the *T. cruzi* strains from DTU TcV, with lower detection signal when using GrO as described (Diez et al., 2010). Primers Grp1 amplified CL Brener, V195 cl1, and CHE, while primers Tul11 allowed detection of Tul cl2. Then, with these two set of primers we could detect all the TcVI reference strains evaluated (Table 4). MLS-PCR of some of the reference strains evaluated are shown in Fig. 1.

Table 4
MLS-PCR with primers X6I, X7I, IB1, Tul11, GrO, GrP, and Grp1 in twenty two reference strains of all *T. cruzi* DTUs.

DTU	Reference strain	X6I	X7I	IB1	GrO	GrP	Tul11	Grp1
TcI	Sylvio X10 cl1	+	+	−	−	−	−	−
	sp104 cl1	+	+	−	−	−	−	−
	sp31	−	−	−	−	−	−	−
	spAI	+	−	−	−	−	−	−
	LQ	−	−	−	−	−	−	−
	13,379 cl7	+	+	−	−	−	−	−
TcII	CBB cl3	−	−	+	−	−	−	−
	Tu18 cl2	−	−	+	−	−	−	−
	IVV cl4	−	−	−	−	−	−	−
	vTV	−	−	+	−	−	−	−
TcIII	M 5631 cl5	−	−	−	−	−	−	−
	P109 cl2	−	−	−	−	−	−	−
TcIV	CAN III cl1	−	−	−	−	−	−	−
TcV	NR cl3	−	−	−	+	+	−	−
	XHCH 56	−	−	−	+	+	−	−
	JGG	−	−	−	+	+	−	−
	SC43 cl1	−	−	−	+	+	−	−
	MN cl2	−	−	−	+	+	−	−
TcVI	Cl Brener	−	−	−	−	−	−	+
	Tul cl2	−	−	−	−	−	+	−
	V195 cl1	−	−	−	−	−	−	+
	CHE	−	−	−	−	−	−	+

3.5. Genotyping in blood samples

Finally, we used the MLS-PCR to genotype *T. cruzi* in 32 blood samples of infected people. As mentioned, amplified products with X61 always showed higher intensity bands than X71. For this reason, we decided to use only this pair of primers to genotype TcI. The same approach was used with GrP instead of GrO for TcV genotyping. Therefore, MLS-PCR was performed with five pair of primers: X61 for TcI, IB1 for TcII, GrP for TcV, and Grp1 and Tul11 for TcV. Fig. 2 shows representative results of some blood samples where a single DTU was detected; and one sample where two different DTUs were detected. In parallel, we genotyped those samples by hybridization tests, and the results with both methods were compared. For just this particular purpose, in mixed detections of two or three different DTUs, we considered them as two or three different samples. Comparative results are shown in Table 5. Only 2 blood samples could not be genotyped by the MLS-PCR assay neither by the hybridization tests. The concordance between both techniques was very good ($\kappa = 0.855$).

Percentages of single and mixed DTUs identified with MLS-PCR are shown in Fig. 3. We detected TcV in 19/30 samples, but only in 9 of them as unique DTU. In the other 10 samples we also identified TcII and/or TcVI. Moreover we detected TcVI in 16/30 samples, in 5 of them as single DTU and in the other 11 together with TcII and TcV. We also detected TcII in 8/30 samples, in 3 of them as single DTU. We should highlight that in 1/30 sample we detected the mixture of TcII, TcV and TcVI together. Finally, we only identified TcI in one sample as single DTU. As a whole, in 18 samples we identified a unique *T. cruzi* DTU; whereas in 12 samples we detected mixture of DTUs (Two samples were not genotyped). Similar results were obtained with hybridization tests using the whole mixture of minicircle sequences as probes (Table 5).

4. Discussion

Numerous approaches have been used to characterize the genetic diversity of *T. cruzi* isolates, mainly based on nuclear markers. However, no single genetic target allows complete *T. cruzi* DTU resolution (Zingales et al., 2012), probably due to the low number of copies of these markers/parasite. In this regard, it is noteworthy that kDNA minicircle markers are present in a much larger number of copies (Wincker et al., 1994). As previously described, *T. cruzi* kDNA forms a complex network of concatenated maxi and minicircles, composed by 10,000–20,000 minicircle molecules/parasite, which increases considerably *T. cruzi* detection sensitivity. Sturm et al. designed specific oligonucleotide primers to amplify a 330 bp fragment from mHVR, hybridizing with the adjacent conserved regions (Sturm et al., 2003).

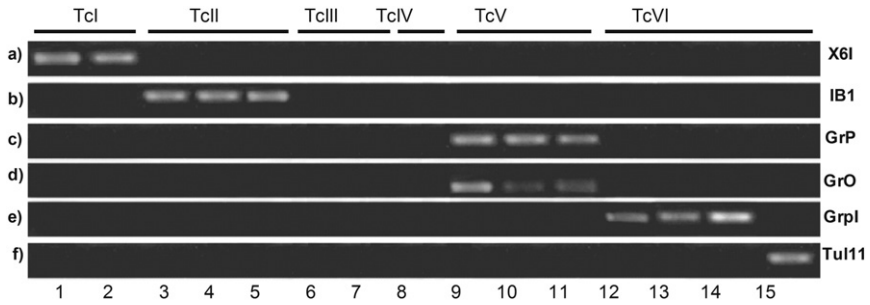

Fig. 1. MLS-PCR for TcI, TcII, TcV, and TcVI. Oligonucleotide pairs: a) X6I, b) IB1, c) GrP, d) GrO, e) Grp1, and, f) Tul11. Strains: 1 — Sylvio X10 cl1; 2 — Sp104 cl1; 3 — CBB cl3; 4 — VTV; 5 — Tu18 cl2; 6 — X109/2; 7 — M5631 cl5; 8 — CAN III cl1; 9 — NR cl3; 10 — JGG; 11 — MN cl2; 12 — ClBrener; 13 — CH2; 14 — V195; 15 — Tula cl2. 1.5% agarose gel stained with EtBr.

Avila et al. have proposed to use these regions to genotype *T. cruzi* (Avila et al., 1991), and since that time different techniques have been developed using kDNA to mention: RFLP or Schizodemes, Low-Stringency Single-Specific-Primer (LSSP)-PCR, and DNA blot hybridization. Previous studies sequencing kinetoplast minicircle showed great heterogeneity within *T. cruzi* (Degrave et al., 1988; Macina et al., 1986; Telleria et al., 2006). This information was used to design genotyping methods using mHVR sequences as DNA probes in hybridization tests (Britto et al., 1995; Macina et al., 1986; Sanchez et al., 1984; Veas et al., 1991). Considering the high mHVR sequence variability, even within the same *T. cruzi* DTU, it is yet unclear why the use of these regions allows discrimination among different *T. cruzi* DTUs. One hypothesis could be the presence of representative sequences within the heterogeneous population of each DTU. Therefore, the use of these sequences may be valuable for *T. cruzi* genotyping.

In this regard, when sequencing minicircle clones from the CL Brener strain we found one major minicircle sequence, partially responsible for the DTU TcVI pattern (Velazquez et al., 2008). Moreover, two major minicircle sequences from DTU TcV were also found when sequencing mHVR from this *T. cruzi* DTU (Telleria et al., 2006). With these TcV and TcVI major sequences, we previously developed a nested PCR (MLS-PCR) to genotype both *T. cruzi* DTUs, and evaluated them on reference *T. cruzi* strains and blood samples from chagasic people from North-Eastern Argentina (Diez et al., 2010).

The aim of this work was to extend the usefulness of the MLS-PCR method to complete the genotyping of the four major *T. cruzi* DTUs found in infected subjects, and to improve detection of the TcVI DTU. With our results, we could say what is ruling the strong signal observed in hybridization tests performed on cloned mHVR sequences is the copy number of these sequences within the probe. Nevertheless, unlike DTUs TcV and TcVI, a low percentage identity among minicircle sequences of the same strain was observed. These results agree with those reported, who have been unable to find predominant minicircle sequences for DTUs TcI and TcII (Telleria et al., 2006).

Analysis in Blast database showed high identity of TcII1 and TcVI1 with reported sequences (Telleria et al., 2006). In that work they defined five groups of predominant sequences within TcVI mHVR: R, S, T, U and V. While group G1 previously reported (Velazquez et al., 2008) showed high identity with group V, TcVI1 identified in this study showed 99% identity with Group U. The analyzed sequences could represent major groups or minicircle classes within TcVI of *T. cruzi*. With the remaining analyzed sequences from these *T. cruzi* DTUs we found low percentage identity compared with those indexed in GenBank, although we should note that at present there are relatively few *T. cruzi* minicircle sequences available. Then, sequences selected for primers design were those with the highest percentage identity with those reported from related *T. cruzi* DTUs.

Results of RFLP and RAPD within TcI showed high heterogeneity within this *T. cruzi* DTU (Luna-Marin et al., 2009). In recent years, many researchers have attempted to sub classify TcI. It has been proposed at least three groups within TcI (O'Connor et al., 2007), while 4 haplotypes have been identified in *T. cruzi* strains of Colombia (Falla et al., 2009; Herrera et al., 2007). TcI reference strains used in those studies are genetically diverse as determined by isoenzymatic analyses, minicircle hybridization tests, and cytochrome *b* gene sequencing (Arenas et al., 2012; Breniere et al., 1991). Given the low identity also found in TcI minicircle sequences analyzed in our work, either among

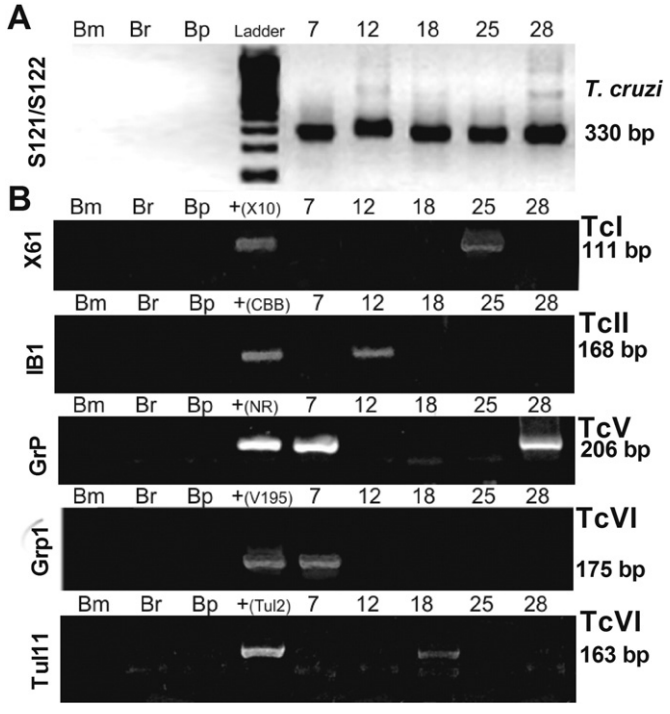

Fig. 2. MLS-PCR in blood samples. Examples of *T. cruzi* genotyping in blood samples of chronic infected people. A) First PCR with S121-S122 primers. B) Nested PCR with MLS-PCR primers. In samples 12, 18, 25 and 28, MLS-PCR was able to identify DTUs TcII, TcVI, TcI, and TcV, respectively. In sample 7 were identified two DTUs (TcV and TcVI). In each assay, the corresponding reference strain was included as positive control. Bm. Sample loading control (water); Br. Reaction control (water); Bp. Purification control (purified water). 1.5% agarose gel stained with EtBr.

Table 5
Comparison of MLS-PCR versus hybridization probes for genotyping of *T. cruzi* in blood samples.

	MLS-PCR					
Hybridization with probes	TCI	TCII	TCV	TCVI	NA[a]	TOTAL
TCI	1	0	0	0	0	1
TCII	0	8	0	0	1	9
TCV	0	0	18	0	2	20
TCVI	0	0	0	15	0	15
NH[b]	0	0	1	1	2	4
TOTAL	1	8	19	16	5	49

[a] NA: no amplification.
[b] NH: no hybridization.

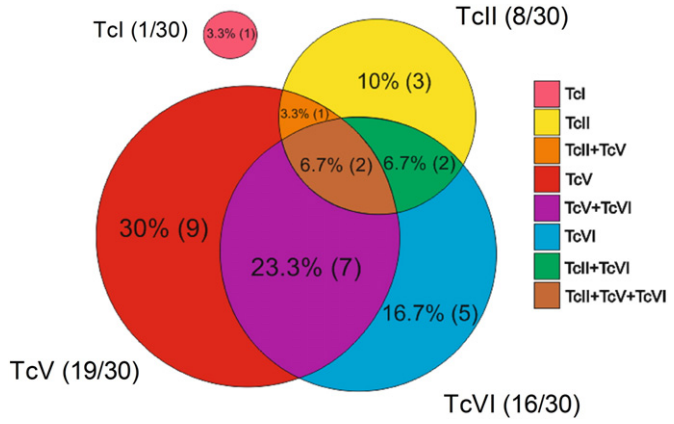

Fig. 3. Distribution of *T. cruzi* DTU in human samples. Percentages of single and mixed detections with MLS-PCR. Numbers inside brackets indicate infected people.

themselves or with those indexed, the criteria adopted for primers design was the use of those with the highest hybridization signal obtained with whole minicircle TcI probes.

Genotyping of TcI reference strains with the current developed MLS-PCR method reached 66.6% sensitivity with pair of primers X61 and 50% with X71. These results indicate the need to improve the TcI detection sensitivity, designing new primers to cover the detection of most strains from this heterogeneous *T. cruzi* DTU. For oligonucleotide pair IB1 the results with TcII reference strains showed 75% sensitivity, while with the complement of the Tul11 and Grp1 primers, we could identify 100% of the analyzed TcVI strains. The results with *T. cruzi* TcII also suggest some heterogeneity within this *T. cruzi* DTU. The same was suggested in previous results using cytochrome *b* gene sequencing and kDNA hybridization tests (Arenas et al. 2012). It should be highlighted the high specificity achieved with MLS-PCR, due to the strategy of carefully selecting the minicircle clones with specific hybridization signal and avoiding those with cross reactivity. Such approach would avoid the possible selection of shared sequences with hybrid DTUs. When MLS-PCR was evaluated in 32 blood samples from infected individuals and compared with hybridization tests using whole minicircles from selected *T. cruzi* DTUs as probes, we obtained a very good agreement (kappa 0.855). We should highlight that with both tests we were able to genotype 94% of the random selected samples analyzed. MLS-PCR-typing showed a predominance of DTU TcV in 63% cases, which is consistent with other studies reported in Argentina and neighbouring countries like Chile and Bolivia (Burgos et al., 2007; Corrales et al., 2009; Diez et al., 2010; Monje-Rumi et al., 2015; Solari et al., 2001; Virreira et al., 2006). Although a smaller proportion of TcVI was found, it was higher than that reported in other studies of Argentina, where it was found associated with dog infections (Diosque et al., 2003; Monje-Rumi et al., 2015), and also indentified in high proportion in triatomines of domicile (Cardinal et al., 2008). Regarding DTU TcII, we have detected it in 27% of the cases, much higher than results reported by other groups. It should be noted that previous studies were performed in different geographical regions, even though all from Argentina. Furthermore, the highest proportion of this *T. cruzi* DTU was detected together with other DTUs. Finally, TcI was only identified in one sample, in agreement with results reported by the above-mentioned researchers from Argentina (Burgos et al., 2007; Monje-Rumi et al., 2015). The fact of the detection of multiple DTUs/sample with this new approach could suggest the presence of mixed infections. With regard to this type of infection, Breniere et al. found that a great heterogeneity of *T. cruzi* populations identified in infected people was inversely proportional to age, similar to previous results from our group (Breniere et al., 2002; Diez et al., 2010). However, in this work, in which all samples were from adults in chronic phase of the disease, we have detected a large proportion of mixed DTUs. The existence of mixed populations in human samples has also been reported by other researchers (Burgos et al., 2007; Flores-Chavez et al., 2006; Monje-Rumi et al., 2015). The use of direct amplification from blood samples without the need for prior isolation and culture of the parasite, could improve detection of such infections.

To previously reported primers for TcV and TcVI genotyping, we added three pair of primers, two of them to genotype TcI and TcII, and the other to improve detection of TcVI. With five pair of primers, we developed a simple methodology to identify major *T. cruzi* DTUs in single and mixed infections directly from human samples.

Conflict of interest statement

None of the authors have a conflict of interest in relation to the content of the present work.

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