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Trypanosoma cruzi: An analysis of the minicircle hypervariable regions diversity and its influence on strain typing

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kDNA, kinetoplastid dideoxy nucleotide acid
mHVR, minicircle hypervariable regions
PCR, polimerase chain reaction
RFLP, restriction fragment length polymorphism
RAPD, randomly amplified polymorphic DNA
LSSP-PCR, low stringency single specific primer polymerase chain reaction
dNTP, deoxynucleotide triphosphate

ABSTRACT

The current intraspecific nomenclature in *Trypanosoma cruzi* describes two major lineages, named *T. cruzi* I and *T. cruzi* II, and five sublineages within *T. cruzi* II, named IIa, IIb, IIc, IId and IIe. The polymorphism of minicircle hypervariable regions (mHVRs) of *T. cruzi* has been used in many studies for the molecular characterization of parasite populations directly from biological samples. However, the molecular bases that allow strain typing by these markers are still unclear. In this work we examined forty cloned mHVRs sequences of CL-Brener reference strain (IIe sublineage), and we found a predominant group of sequences, with 40% of frequency in this strain, with a 97% of identity among them. Out of the forty clones analyzed, we identified other less representative types, and a few unique ones. This predominant sequence is also present in different reference strains belonging to the other main *T. cruzi* lineages and sublineages (TcI, IIa, IIb, IIc and IId) although in a many thousand times lower frequency than in the CL-Brener strain, as shown by semiquantitative PCR. Similarly, predominant mHVR sequences previously described for TcIId strains, were clearly more frequent (many thousand times higher) in the IId reference strain analyzed by us (Mncl2) than within the reference strains belonging to the other lineages and sublineages. The analysis of the cloned sequences shows that more sequences than just the major one contribute to define the global pattern of mHVRs RFLP in the CL-Brener strain. The possible usefulness of these predominant sequences for typing TcIId and TcIIe sublineages by semiquantitative PCR, as well as the possible role of these sequences in genotype identification by mHVR probes are discussed.

1. Introduction

Trypanosoma cruzi, the chagas disease etiologic agent, infects between 16–18 million people in Latin America (Moncayo, 2003). The infection affects individuals both during the acute and the chronic phases of the disease, with different degrees of severity. During the acute phase, there are few cases with clinical manifestations, in spite of the large number of parasites that are present in peripheral blood. However, in the acute phase, the infection may be fatal in a few cases. A variable number of years after the initial infection, 27% of the infected people develop cardiac symptoms which may lead to sudden death, 6% develop digestive damage, mainly megaviscera, and 3% peripheral nervous system involvement (Moncayo, 2003). The diverse manifestations mentioned above may be explained in part by the wide genetic heterogeneity of *T. cruzi*. This idea is strengthened by the differences in virulence and tropism of the strains, which were associated with genetic heterogeneity (Risso et al., 2004; Vago et al., 1996a,b, 2000). Moreover, in the human illness, different genotypes of the parasite have been related to virulence degree or antibiotic resistance (Breniere et al., 1998; Coronado et al., 2006).

These findings stress the importance of furthering knowledge on the infecting strains to determine whether there exist associations between strains and clinical features and, eventually, to develop specific prognoses and treatments according to each clinical case. Different isoenzymatic and genetic analyses led an expert committee to designate two highly divergent major lineages as *T. cruzi* I (TcI) and *T. cruzi* II (TcII) (Anonymous, 1999). Moreover, *T cruzi* II is a very heterogeneous group (Tibayrenc, 1995; Souto et al., 1996). This lineage could be partitioned in five sublineages (TcIIa–e) based on isoenzymes and RAPD typing (Brisse et al., 2000, 2001). This classification is mainly based on techniques requiring parasite isolation.

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As the isolation of the parasites in cultures or mice could lead to strain selection (Devera et al., 2003), only direct characterization from insects and mammalian samples allow strains to be typed without bias. On this basis, and given the high sensitivity of minicircle amplification by PCR, the polymorphism of minicircle hypervariable regions (mHVRs) has been used in many studies for the molecular characterization of parasitic populations directly from biological samples. Minicircles are circular DNA present in every member of the Kinetoplastid order and organized in an extensive Kinetoplast DNA network. These molecules codify gRNAs which are responsible for cryptogene editing by U-insertion deletion in mRNA. Moreover, it has been suggested that many minicircles have a role in maintaining a certain genome size and topology of the network (Lukes et al., 2005). The size and structure of minicircles are species-specific. *T. cruzi* minicircles have 1.4 kbp and are composed of four conserved regions and four hypervariable regions which codify gRNA and are responsible for the heterogeneity observed in different strains (Degrave et al., 1988). Amplified mHVRs with primers designed from flanking conserved regions are used as PCR product to strain typing by RFLP analysis or hybridization probes (Jaramillo et al., 1999; Rodriguez et al., 2002; Bastrenta et al., 1999; Morel et al., 1980; Sturm et al., 1989; Britto et al., 1995). The mHVR were also used to distinguish different strains by LSSP-PCR (Vago et al., 1996a,b, 2000; Franco et al., 2003 and Salazar et al., 2006). The specificity obtained is not clearly explained by the sequences described for mHVRs, since low levels of similarity were observed not only between minicircle sequences of different strains (Telleria et al., 2006) but also within the same minicircle, and among different minicircles of the same kDNA network (Degrave et al., 1988). However, Telleria et al. (2006) showed that some sequences are amplified repeatedly from each strain, and that these sequences appear only in strains of the same sublineages. This comparison was done on the products obtained by amplifying and cloning some mHVR sequences from different *T. cruzi* strains.

Based on these results it may be suggested that the lineage specific pattern is determined by the presence of predominant mHVR sequences in each different lineage or due to global sequence diversity, as a fingerprinting. To define one of these hypothesis we have raised and answered these questions: (i) is the predominance of some sequences kept in repeated PCRs from a particular strain? (ii) are these predominant sequences lineage-specific? and (iii) are these sequences responsible for the RFLP pattern features?

2. Materials and methods

2.1. Parasites and sample preparation

Cloning and sequencing were performed on CL-Brener strain (sublinage TcIIe) and semiquantitative PCR was performed on CL-Brener and reference strains belonging to the other five main lineages and sublineages of *T. cruzi*, namely: X10cl1 (TcI), CANIIIcl1 (TcIIa), TU18cl93 (TcIIb), M5631cl5 (TcIIc) and MNcl2 (TcIId). All the strains were cultured in liver infusion tryptose (LIT) at 28 °C. Each stock was harvested by centrifugation (2800*g*, 20 min, 4 °C) and washed in PBS. Cells were resuspended in 100 mM Tris–HCl, pH 8.0, 400 mM NaCl, 10 mM EDTA-Na_2, and 1%, SDS, and samples were incubated for 2 h at 37 °C in the presence of 100 $\mu g\,mL^{-1}$ RNase A, and then kept overnight at 55 °C with 200 $\mu g\,mL^{-1}$ proteinase K. DNA purification was carried out by phenol–chloroform extraction and ethanol precipitation.

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

Segments of 330-bp kinetoplast DNA 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′). 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), 3 mM $MgCl_2$, 1.5 IU Taq DNA polymerase (Invitrogen), 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 30 cycles of 1 min at 94 °C, 1 min at 59 °C and 1 min at 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.

2.3. Cloning and sequencing

The PCR products were purified with a Wizard SV Gel and PCR Clean-Up System kit purification (Promega). The CL-Brener-amplified fragments from four different PCRs were cloned using the pGEM T easy vector (Promega) in the fragment: vector molar ratio indicated by the manufacturer. Ten clones of each PCR product were randomly selected, and sequenced by MACROGEN Inc. (Automated Sequencing Service).

2.4. Sequence analysis

Nucleotide sequences were deposited in the GenBank database with Accession Nos. EU088377–EU088401. The sequences were aligned to each other using the edit sequence-DNA Star program version 3.01, and BioEdit Sequence Alignment Editor, version 7.0.5. All sequences were analysed for similarity rate among them and with any previously-indexed kDNA sequence of *T. cruzi*, using the BLAST algorithm.

2.5. Statistical analysis

To estimate the proportion of repeated sequences in the total population, the 95% confidence interval of the sample proportion was calculated (Cochran, 1977). This analysis was performed considering that (i) each parasite kinetoplast contains 30,000 minicircles with four mHVRs each (Degrave et al., 1988), and (ii) the amount of each different mHVR sequence, at all PCR amplifications, is representative of the natural proportion of these kinetoplast sequences. Therefore, we estimated 120,000 different, possible sequences present in the purified kDNA of any *T. cruzi* strain. The distribution of sequences in each PCR was analysed by the chi-square test, using the R free software (http://www.r-project.org/).

2.6. Semiquantitative PCR

The relative amount of the major sequence identified in this work within CL-Brener-mHVRs, was quantified in different strains by a semiquantitative PCR adapted from Holman et al. (2003) and Bathelier et al. (1996). First, the 330 bp-PCR products of each strain were quantified as mentioned below. pGEM-T easy vector DNA containing an insert of the major CL-Brener-mHVR sequence was quantified by densitometry, at 260 nm. The proportional quantity of the released 330 bp-PCR product was calculated, after digestion with EcoRI. Then, the PCR products from each strain were quantified by comparison of the electrophoresis gel images intensity obtained by digital capture with the DNA insert intensity taken as reference, using the Scion Image analysis software. Afterwards, the amplified products from each strain were diluted from 10^2 to 10^{-9} ng, and all dilutions were then amplified using the primers GrpI Fw (5′-ATCCAGACCCCAA TTTTACTAC-3′) and GrpI Rv (5′-ATGTGATTGGATAGGTGATAGAT 3′), complementary to the ends of the major CL-Brener mHVR

sequence. Reactions were performed in a total volume of 50 μL, containing 250 μM of each dNTP, 0.25 nM of each primer (Invitrogen), 1.5 mM $MgCl_2$ and 1.5 IU Taq DNA polymerase (Invitrogen). The amplification was performed programming an initial denaturation step at 94 °C for 2 min followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a 3 min final extension step at 72 °C. One-tenth of the reaction products was electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Likewise, major sequences previously described in TcIId (Telleria et al., 2006), which correspond altogether to the 80% of total mHVRs, were quantified in different strains. The primers designed to quantify sequence "O" were GrOFw (5′-GGCTTAGGGTGTGGATA GG-3′) and GrO Rv (5′-ATCGCGAAACCCATACAA-3′) and to quantify sequence "P"; GrP Fw (5′-GTTTGCGGTTTATGTTATTGAAT-3′), and GrP Rv (5′-TTCCAACCCCACAAATGATA-3′). The cycles and temperatures were similar to those previously described for G1.

2.7. RFLP

The amplified 330 bp mHVRs (10 μg mL^{-1} of DNA) were digested with 3 IU Mse I, 2 h at 37 °C. Five microliter of the DNA fragments produced in each digestion were separated on a 10% polyacrylamide gel prepared with 10% (w/v) acrylamide/bisacrylamide (38:2) and visualized with 0,5 μg mL^{-1} ethidium bromide.

3. Results

3.1. PCR, cloning, and sequence analysis

Four PCRs were carried out from the total DNA of the CL-Brener strain (PCR A, PCR B, PCR C and PCR D) to amplify the mHVRs. Each PCR product was cloned in T easy vector plasmid (Promega) and 10 clones were randomly sequenced from PCR A, PCR B, PCR C and PCR D, respectively. When high identity was observed between sequences they were grouped. The identity within groups was in every case superior to 97% (Fig. 1) and between groups inferior to 47%. The groups were arbitrarily named GI, GII, GIII, GIV, GV, GVI, GVII and GVIII (Table 1). We found a major sequence with a 40% of predominance (GI), the relative-frequency interval being 24–56% (0.95 CI, $N = 120{,}000$, $n = 40$, and $S_{\text{proportions}} = 16$). Other sequences appeared less frequently, namely: GII (7.5%), GIII, GIV, GV, GVI, GVII and GVIII (5%). About twenty percent (22.5%) of the analysed sequences were classified as "unique sequences". These unique sequences showed different sizes, the highest difference being 90 bp.

We observed a homogeneous proportion of GI sequences in each of the four PCR ($\chi^2 = 5.83$; $p = 0.120$). However, the homogeneity of other groups could not be analysed because many of the bivariate table values had frequency 0.

The comparison between our sequences and those reported in the GenBank was performed using BLAST and it is summarized in Table 2. We found a reported sequence of CL-Brener-mHVRs with high identity to GI. Group III and Group VII also showed high identity to other CL-Brener sequences and GIV showed high identity to a x154/7 strain sequence (TcIIe), all of these reported by Telleria et al. (2006), whereas GV and GVI showed high identity to other CL-Brener sequences previously reported by Degrave et al. (1988). Two of the unique sequences, B2 and A7, also showed identity to a CL-Brener and an x154/7 strain sequence, respectively, and another one (D9), with an X10 cl1 strain sequence, belonging to the phylogenetically-distant *T. cruzi* I lineage, reported by Telleria et al. (2006). Two groups (GII and GVIII) and five unique sequences (B10, C4, C7, D4, and D5) did not match any reported sequences (Table 2).

3.2. Semiquantification of major CL-Brener and TcIId mHVR sequences in different lineages

To determine if the major sequence is specific of the CL-Brener strain or whether it is also present in other strains, we designed primers to amplify GI using as template increasing dilutions of mHVR PCR products from the reference strains. By doing so, we were able to determine the presence of this sequence in every strain. However, it was less represented in strains different from CL-Brener; the detection limit was 10 ng for X10 cl1 (TcI), 1 ng for CAN III cl1 (TcIIa), 1 ng for TU 18 cl93 (TcIIb), 1 ng for M5631 cl5 (TC IIc), 0,1 ng for Mn cl2 (TcIId) and 10^{-8} ng for CL-Brener (TcIIe). Interestingly, we found that the higher the phylogenetic distance between CL-Brener and the other strains, the lesser the level of occurrence of the sequence.

We performed the same analysis with sequences "O" and "P" described as major sequences for TcIId sublinage (Telleria et al., 2006). In this case the detection limit of the "O" sequence was 1 ng for X10cl1 (TcI), 10 ng for CAN III cl1 (TcIIa), 0,1 ng for TU 18 cl93 (TcIIb), 10 ng for M5631 cl5 (TcIIc), 10^{-8} ng for Mn cl2 (TcIId) and 1 ng for CL-Brener (TcIIe). The detection limit of the "P" sequence was 1 ng for X10cl1 (TcI), 1 ng for CAN III cl1 (TcIIa), 1 ng for TU 18 cl93 (TcIIb), 1 ng for M5631 cl5 (TcIIc), 10^{-8} ng for Mn cl2 (TcIId) and 0,1 ng for CL-Brener (TcIIe).

3.3. RFLP profiles

In order to ascertain whether the major sequence produces a strain-characteristic-RFLP pattern, we selected the MseI restriction enzyme, whose restriction site is present in all the sequences obtained. The PCR products obtained from direct amplification of mHVRs, and the 40 cloned mHVR sequences, were digested with this enzyme. As expected, clones of the same group have the same RFLP pattern and clones of different groups and unique sequences showed different RFLP patterns (Fig. 2). The patterns were coincident with the predicted patterns based in the sequences. CL-Brener-total-mHVR pattern had 9 bands that we have called 1–9 as they appeared from the top to the bottom of the Gel, respectively. When the GI RFLP pattern was compared with the CL-Brener-total-mHVR pattern, in both profiles 3 bands with the same length were identified (bands 4, 6 and 9). The CL-Brener-total-mHVR pattern also shared 3 bands with GII (bands 6, 7, and 8) and 2 bands with GIII (bands 3 and 7), GIV (bands 6 and 8), GV (bands 3 and 4), GVIII (bands 1 and 9), and with B2 (bands 1 and 2) and C7 (2 and 9) sequences. One common band was identified for sequences GVI (band 2), GVII 8 (band 1), A7 (band 6), B5 (band 1), B10 (band 8) and D9 (band 6), while C4, D4 and C5 had no common bands. These results show that GI sequence would explain 33% (3/9) of CL-Brener-total-mHVR pattern while all the 40 sequences analysed, including G1 would explain 89% (8/9) of CL-Brener-total-mHVR pattern. If only the patterns of repeated sequences (G1–G8 sequences) are considered, also 89% of bands are shared with CL-Brener-total-mHVR pattern. Then, we cannot conclude that GI sequence alone defines the strain pattern but it is likely that a low number of sequences would define it.

4. Discussion

Although several markers have been used to type *T. cruzi* strains directly from biological samples (Marcet et al., 2006), mHVRs have the advantage of their high number of copies present per parasite (Wincker et al., 1994). The reason why these markers allow strain typing is still unclear, but we can advance two different hypotheses: (i) the presence of predominant mHVR sequences in each different lineage determines the lineage specific patterns; or (ii) the

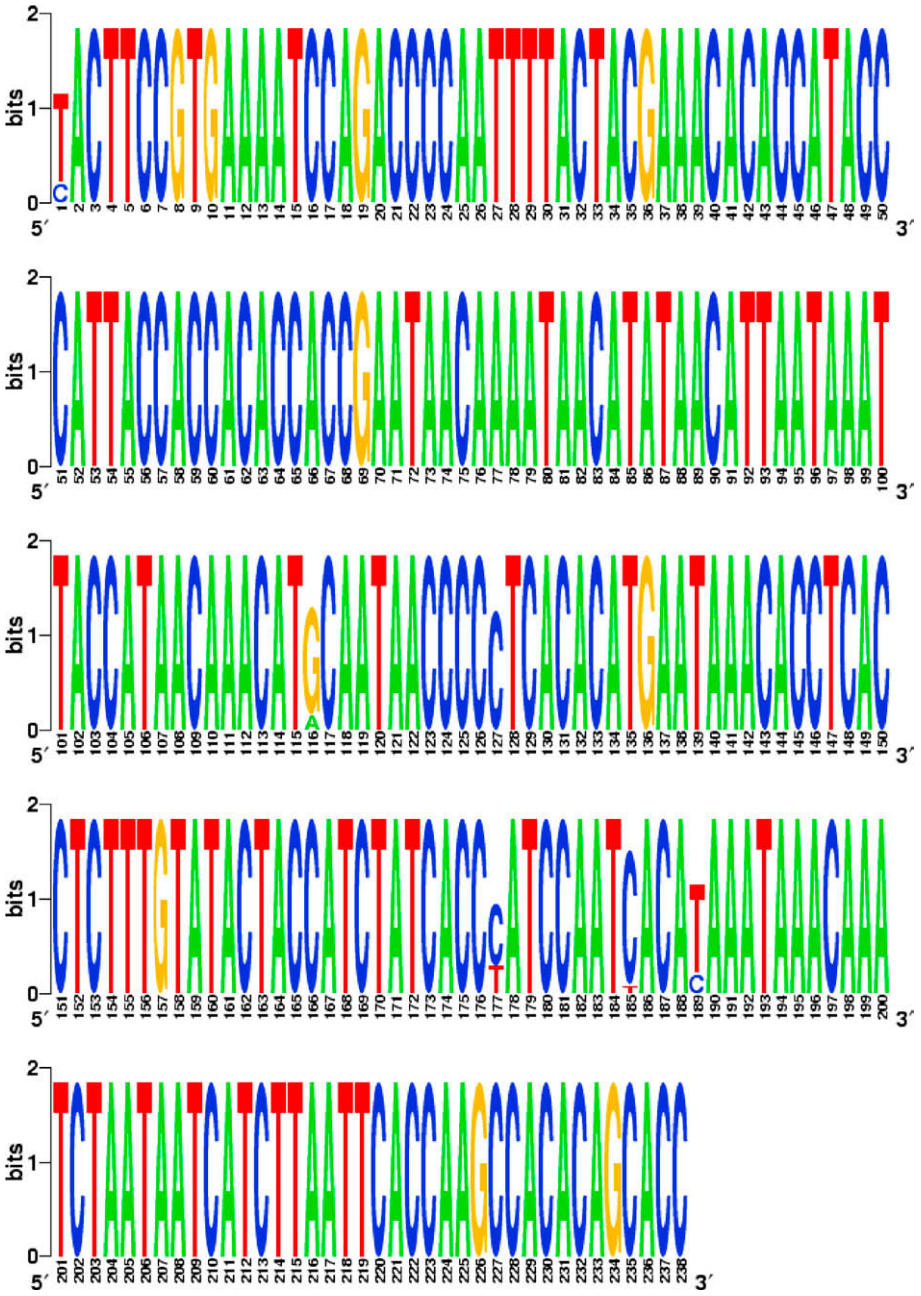

Fig. 1. The Weblogo diagram http://weblogo.berkeley.edu shows the high degree of identity within group 1. At each position the height of the letters represents the proportions with the base represented by the letter.

Table 1
Grouping by sequence identity of clones obtained from 4 PCR products of *T. cruzi* CL-Brener strain mHVR

Name	Sequence number					Identity%
	A	B	C	D	Total	
GI	5	6	4	1	16	97.4
GII	2	0	1	0	3	99.6
GIII	1	0	1	0	2	100
GIV	0	0	1	1	2	100
GV	0	0	1	1	2	100
GVI	0	1	0	1	2	100
GVII	0	0	0	2	2	99.6
GVIII	1	0	0	1	2	100
Unique sequences	1	3	2	3	9	<30

G, group; A, B, C, and D. the four PCRs performed.

specific patterns are due to global sequence diversity, as a single sequence pattern.

To examine these two hypotheses, we have cloned, sequenced and analysed PCR products of the CL-Brener strain. Out of the forty clones analysed, we identified one predominant group of sequences (G1), other less representative types, and a few unique ones.

As we used PCR to obtain mHVR sequences, we wanted to know, first, if this reaction showed the same sample proportion of sequences in each run. This information was important to rule out a technical bias in our results. Then, we analyzed four different reactions and we found that they had a similar G1 ratio. Based on these data, we can assume the reproducibility of PCR regarding G1 amplification.

Table 2
Comparison between obtained and reported *T. cruzi* mHVR sequences

	Name	Access No.[a]	"*E*" value[b]	Strain (lineage)
Grouped sequences	GI	AJ748040.1	6.00E-54	CL-Brener (TCIIe)
	GII	–	—	—
	GIII	AJ748037.1	7.00E-57	CL-Brener (TCIIe)
	GIV	AJ748065.1	6.00E-122	x154/7 (TCIIe)
	GV	M18816.1	1.00E-85	CL-Brener (TCIIe)
	GVI	M18816.1	2.00E-121	CL-Brener (TCIIe)
	GVII	AJ748039.1	6.00E-125	CL-Brener (TCIIe)
	GVIII	—	—	—
Unique sequences	A7	AJ748064.1	2.00E-121	x154/7 (TCIIe)
	B2	AJ748041.1	1.00E-45	CL-Brener (TCIIe)
	B10	—	—	—
	C4	—	—	—
	D4	—	—	—
	D5	—	—	—
	D9	AJ747962.1	5.00E-70	X10 cl1Z1 (TCI)

Hyphens were placed when no identity was found.
[a] GenBank Accession No. of previously-indexed sequences.
[b] Highest *E* values for grouped sequences, obtained by comparing each group sequence with the one indexed.

Our finding of a predominant mHVR in a strain has already been described in other kinetoplastid species. In the C1 strain of *Crithidia fasciculata*, for example, a 90% predominant minicircle was determined by specific probes hybridation (Yasuhira and Simpson, 1995). In *Leishmania tarentolae*, it has been described a high abundant minicircle class estimated to be 25% of the total mHVRs (Maslov and Simpson, 1992). Also in mHVR amplified from a patient infected with *Leishmania donovani*, 75% of the obtained sequences were highly conserved (Singh et al., 1999). In *T. cruzi*, similar analyses were previously done in representative strains of each lineage (Telleria et al., 2006). These authors analysed three up to 14 sequences from some representative strains of lineages I, and sublineages IIb, IId and IIe. Then, a total of 35 sequences were analyzed for different strains of sublineage TcIIe and five predominant sequences were found corresponding to T:17.1%; R:5.7%; S:5.7%; U:11.4%; and V:13.5%, of the total sublineage (the nomenclature correspond to these used by the authors). In TcIId, 90% of sequences corresponded to only three classes of sequences (O: 43%; P: 37%; and Q: 10.8%). With respect to lineages TcI and sublineage TcIIb, Telleria et al. (2006) did not found major sequences, however it would be necessary to increase the amount of sequences analysed per strain to determine if anyone is present in more proportion. If major sequences were found in other lineages and sublinages different than TcIId and e, it would be interesting to analyze these sequences in distant phylogenetic strains.

In our work, we aimed at analyzing a higher number of sequences of the CL-Brener strain. We found that most of our repeated sequences, and some unique sequences, were the same as those reported by Telleria et al. (2006) for CL-Brener and other TcIIe strains. The sequence with high identity to G1 was observed in two out of the seven CL-Brener sequences analysed in the cited work. A striking result is that one of our clones was reported in a lineage corresponding to TcI in GenBank reported sequences. It is worth noting that Cribb et al. (2004) reported for CL-Brener strain, the presence of "*T. cruzi* I-like" sequences for the spliced leader RNA gene promoter, and propose that these sequences could be considered a molecular trace of a hybrid origin and a new evidence for the presence of sequences of *T. cruzi* I origin into a *T. cruzi* II strain. A similar situation was reported in *L. tarentolae* in which a minicircle sequence previously reported in a phylogenetic distant *Leishmania* strain was found (Singh et al., 1999). However, this result is not in agreement with those obtained by Telleria et al. (2006). These authors found no sequence classes shared among different phylogenetic groups of *T. cruzi*. Hence, these authors claimed that specific characterization of closed phylogenetically related genotypes with probes or RFLP mHVR patterns, should correspond, at the sequence level, to the existence of genotype-specific sequences families and to the near-absence of common sequences among different phylogenetic groups of the parasites. As the absence of G1 sequence after cloning and sequencing the mHVR of a strain do not guarantee the absence of this sequence, even by checking a high number of clones, we used the PCR approach to evaluate whether the major sequence is sublineage-specific or is also present in other lineages or sublineages.

Semiquantitative PCR, based on the limit of sensitivity of this technique, allowed us to determine that G1 sequence occurs at least 10^{-7} times less frequently in TcIId, and even less frequently in more distant lineages as TcI. Then, we observed that the longer phylogenetic distance from CL Brener strain, the lower concentration of G1. These results should be confirmed in several strains of each phylogenital group of *T. cruzi*, however they are compatible with the current knowledge of this taxon (Brisse et al., 2000, 2001; Westenberger et al., 2005, 2006; de Freitas et al., 2006).

Taking into account that *T. cruzi* multiplication seems to be clonal with some rare sexual exchanges along the evolution (Tibayrenc et al., 1986; Westenberger et al., 2006) and that trypanosomatids minicircles would be transmitted by random segregation (Thiemann et al., 1994; Savill and Higgs, 1999), the

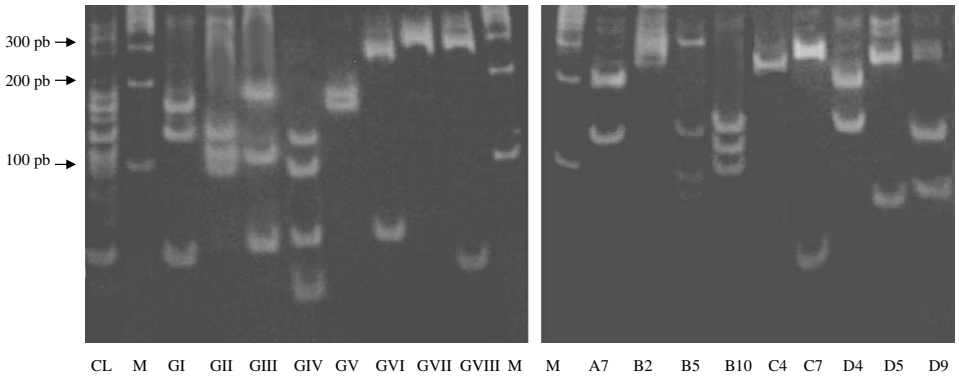

Fig. 2. RFLP patterns from total population and cloned CL-Brener amplified mHVR sequences. The MseI digested products were visualized in a polyacrylamide gel stained with ethidium bromide. Lane CL, CL-Brener; Lanes M, 100 bp ladder size marker; GI-GVIII, groups I to VIII, respectively; A7, B2, B5, B10, C4, C7, D4, D5 and D9, unique sequences.

frequencies of some sequences in different lineages and sublineages would be higher in closer phylogenetically related strains than in distant ones. According to our results, we observed that the two hybrid strains that would have diverged most recently, TcIId/e (Brisse et al., 2000, 2001; Westenberger et al., 2005; de Freitas et al., 2006), have closer minicircle populations between them compared to those of the distant TcI.

Defined mHVRs have already been used to type strains. Virreira et al. (2006) used defined probes from two major sequences of TcIId (sequences "O" and "P") to type this sublineage according to the pattern obtained with the probes. They have even proposed the differentiation of three groups within sublineage TcIId. In our work, we have done the same approch that we had performed for G1 sequence to TcIId major sequences and we have found that "O" and "P" sequences were predominant only in TcIId. This finding is in accordance with the hypothesis of presence of characteristic major sequences in different sublineages, and suggests that semiquantitative PCR could be a good tool for typing *T. cruzi* strains by obtaining a cut-off value to differentiate one from the other.

To determine whether the major sequence is responsible for the characteristic RFLP patterns observed in the CL-Brener strain, RFLP profiles from total amplified CL-Brener-mHVR sequences and from each of the forty cloned sequences were obtained. From the results we conclude that, even when a major sequence is responsible for some bands, this does not completely determine the specific pattern observed. This pattern has common bands with most of the cloned sequences and then the global sequence diversity probably determines each sublineage RFLP pattern. The same analysis could be done for probes or LSSP-PCR technique. Probes made from total kDNA for strain typing, using high stringency conditions have been described by Avila et al. (1990). In this early work, as in others in which probes were used for typing different strains, the specific signature could be defined by few predominant sequences present in each strain or by global diversity responsible for the signature. In the present work, we did not found a unique sequence responsible for CL-Brener RFLP pattern, but our results indicate that a limited number of sequences would define it as few sequences have fit with 89% of the total pattern. In the oposite site, when mHVR is used to type strains by hybridization probes, the crossreaction between sublineages could be related to the presence of major sequences of one sublineage present in lesser amount in the other. Recently, Virreira et al. (2006) have typified strains with probes made from TcIId and TcIIe kDNA. These authors found that the TcIIe probe hybridised non-specifically with a TcIIb strain, which can be explained by this assumption.

We can then conclude that in spite of the presence of a major sequence, in the RFLP technique this presence is not solely responsible for the specific pattern. However, using these major sequences as specific markers we were able to perform a semiquantitative PCR approach to type lineages and sublineages. Then, the identification of major lineage specific sequences in other lineages and sublineages would be a useful tool to characterize different infecting strains directly from samples, avoiding the bias introduced by the isolation of micro-organisms. These sequences could be used for PCR, probes or RFLP which should be labelled with specific probes.

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