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Further genetic characterization of the two *Trypanosoma cruzi* Berenice strains (Be-62 and Be-78) isolated from the first human case of Chagas disease (Chagas, 1909)

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Abstract

We describe here an extension of a previous genetic characterization of *Trypanosoma cruzi* strains (Be-62 and Be-78) isolated from the patient Berenice, the first human case of Chagas disease [Chagas, C., 1909. Nova Tripanomíase humana. Estudos sobre morfologia e o ciclo evolutivo do *Schizotrypanum cruzi*, n. gen., n. sp., agente etiolójico da nova entidade morbida do homem. Mem. Inst. Oswaldo Cruz 1, 159–218]. We wanted to verify the composition of *T. cruzi* populations originated from these two isolates. In the present work, 22 enzymatic loci (MLEE), nine RAPD primers and 7 microsatellite loci were analyzed. Clones from both strains were also characterized to verify whether these strains are mono or polyclonal. Be-62 and Be-78 strains were different in 3 out of 22 enzymatic systems, in 3 out of 9 RAPD primers tested and in all microsatellite loci investigated. However, our data suggests that both strains are phylogenetically closely related, belonging to genetic group 32 from Tibayrenc and Ayala [Tibayrenc, M., Ayala, F.J., 1988. Isoenzime variability in *Trypanosoma cruzi*, the agent of Chagas' disease: genetical, taxonomical, and epidemiological significance. Evolution 42, 277–292], equivalent to zymodeme 2 and *T. cruzi* II major lineage which, in Brazil, comprises parasites from the domestic cycle of the disease. Microsatellite analyses showed differences between

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the parental strains but suggested that both populations are monoclonal since each strain and their respective clones showed the same amplification products.

Keywords: *Trypanosoma cruzi*; Berenice strains; Isoenzyme; RAPD; Microssatellites

1. Introduction

Chagas disease is a very important tropical disease in South America caused by *Trypanosoma cruzi*. The first human case of was discovered by Chagas (1909) when he examined a 2-year-old girl, named Berenice, in Lassance, Minas Gerais state, Brazil. Two strains of *T. cruzi* isolated from this patient in 1962 and 1978 and further named Be-62 (Salgado et al., 1962) and Be-78 (Lana and Chiari, 1986) were comparatively studied revealing several important differences. Be-78 showed to be different from Be-62 in relation to morphology, parasitemia, tissue tropism, pathogenicity, mortality in mice, growth and differentiation in LIT media (Lana and Chiari, 1986) and izoenzyme and kDNA profiles (Lana et al., 1996). Furthermore, contrasting with Be-62 infections, mice and dogs infected with Be-78 develop intense cardiac lesions and fibrosis reproducing experimentally the characteristics of the human chagasic cardiopathy (Lana and Chiari, 1986; Lana et al., 1992; Araújo et al., 2002). However, from the molecular point of view, especially in relation to nuclear genome, these strains had been poorly studied. To further investigate the population structure of Berenice strains we extended the initial biochemical and genetic characterization of them by using extra enzymatic loci (MLEE—multilocus enzyme electrophoresis), randomly amplified polymorphic DNA (RAPD), and microsatellite analyses. In addition, laboratory clones derived from both strains were also characterized.

2. Material and methods

2.1. T. cruzi strains and clones

Be-62 (Salgado et al., 1962) and Be-78 (Lana and Chiari, 1986) strains were isolated by xenodiagnosis from Berenice patient and maintained by successive blood passages in mice. For this study parasites were isolated from mice through hemoculture, cultivated in LIT medium and clones were obtained by successive dilutions according to Gomes et al. (1991).

The parental strains (Be-62 and Be-78) and their respective clones (Be-62cl3, Be-62cl7, Be-62cl8 and Be-62cl12) and (Be-78cl2, Be-78cl9, Be-78cl10 and Be-78cl15) were characterized. We also analyzed, as a reference, *T. cruzi* clones from the main natural genetic groups or clonets (Tibayrenc and Ayala, 1988) including SilvioX10cl1 (19–20), CANIIIcl1 (27), MAS1cl1 (32), MNcl2 (39), M6241cl5 (36) and TULAcl2 (43).

2.2. Multiloci enzyme electrophoresis (MLEE) analyses

For isoenzyme analyses a volume of approximately 80 ml of parasites in exponential growth phase in LIT medium was used. Flagellates were washed twice with phosphate buffer solution (PBS) by centrifugation at 3500 rpm, 4 °C, and the pellet stored at −70 °C until use. Enzymatic extracts were prepared according to Kilgour and Godfrey (1973) and cryopreserved in liquid nitrogen.

The enzymatic extracts were analyzed in cellulose acetate plates according to Ben Abderrazak et al. (1993). Twenty enzymes were studied: aconitase (E.C. 4.2.1.3, ACON), alanine aminotransferase (E.C. 2.6.1.2, ALAT), diaphorase (E.C. 1.6.99.2, DIA), glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12, GAPD), glutamate dehydrogenase NAD+ (E.C. 1.4.1.2, GDH-NAD+), glutamate dehydrogenase NADP+ (E.C. 1.4.1.4, GDH-NADP+), aspartate amino transferase (E.C. 2.6.1.1, GOT), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, G6PD), glucose-6-phosphate isomerase (E.C. 5.3.1.9, GPI), isocitrate dehydrogenase (E.C. 1.1.1.42, IDH), leucine aminopeptidase (cytosol aminopeptidase) (E.C. 3.4.11.1, LAP), malate dehydrogenase (E.C. 1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating, NADP+) or malic enzyme (E.C.

1.1.1.40, ME), mannose-phosphate isomerase (E.C. 5.3.1.8, MPI), nucleoside hydrolase (E.C. 2.4.2.1, NHi); substrate: inosine, peptidase 1 (E.C. 3.4.22.3, formerly E.C. 3.4.4.12, PEP-1); substrate: leucyl-leucyl-leucine, peptidase 2 (E.C. 3.4.22.4, formerly E.C. 3.4.4.24, PEP-2; substrate: leucyl-L-alanine), 6-phosphoglucomutase dehydrogenase (E.C. 1.1.1.44, 6PGD), phosphoglucomutase (E.C. 5.4.2.2, formerly E.C. 2.7.5.1, PGM), and superoxide dismutase (E.C. 1.15.1.1, SOD).

2.3. RAPD analysis

DNA extractions were performed from the pellets remaining after the enzymatic extraction, according to Brisse et al. (2000). Each RAPD reaction was performed in a total volume of 60 μl of 1× Mg-free buffer; containing 0.9 U *Taq* DNA polymerase (GIBCO, Invitrogen); 0.1 mM dNTP mixture (Pharmacia Biotec); 1.5 mM $MgCl_2$; 0.2 μM of each primer and 20 ng of the DNA template. Twenty microlitres of mineral oil was added to the mixture to avoid evaporation of the reagents. Primers F13 (5′-GGCTGCAGAA-3′), B15 (5′-GGAGGGTGTT-3′), N19 (5′-GTCCGTACTG-3′), A10 (5′-GTGATCGCAG-3′), F15 (5′-CCAG-TACTCC-3′), N9 (5′-TGCCGGCTTG-3′), A15 (5′-TTCCGAACCC-3′), U7 (5′-CCTGCTCATC-3′) and B19 (5′-ACCCCCGAAG-3′) from Operon Technologies, Alameda California, CA, USA, were used.

The amplification program comprised 45 cycles: initial denaturation step at 94 °C for 1 min, annealing at 36 °C for 1 min; extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 7 min.

Electrophoresis was performed in 1% agarose gels at 100 V. Products were stained by ethidium bromide and visualized under UV light.

2.4. Microsatellite analyses

Microsatellite analyses were performed with DNA obtained from the parental strains and their clones cultivated in LIT medium. *T. cruzi* stocks were analyzed with the SCLE10 (5′-GATCCCGCAATAGGAAAC-3′ and 5′-GTGCATGTTCCATGGCTT-3′), MCLE01 (5′-CTGCCATGTTTGATCCCT-3′ and 5′-CGTGTA-CATATCGGCAGTG-3′), MCLG10 (5′-AGGAGTC-AAATATAATGAGGCA-3′ and 5′-ACGTGTGAAA-GGCATCTATC-3′) loci described by Oliveira et al. (1998) and TcTAC-02 (5′-GAATTTCCCCATTTCC-AAGC-3′ and 5′-CGATGAGCAACAATCGCTTC-3′) TcAAT-01 (5′-ACCTCATCGGTGTGCATGTC-3′ and 5′-GTCGCCGTGCAATTTC-3′), TcTAT-01 (5′-GATCCTTGAGCAGCCACCAA-3′ and 5′-CAAAT-TCCCAACGCAGCAGC-3′), TcAAAT-01(5′-GCC-GTGTCCTAAAGAGCAAG-3′ and 5′-GGTTTTAG-GGCCTTTAGGTG-3′) loci kindly provided by Dr. Juliana Pimenta. Amplification was performed basically as described by Oliveira et al. (1998). Each reaction was performed in a total volume of 15 μl containing 0.75 U *Taq* DNA polymerase (Phoneutria), 2.5 mM each dNTP and 0.05 μM each primer. The reaction buffer consisted of 100 mM Tris–HCl (pH 8.8), 35 mM $MgCl_2$, 250 mM KCl and 3 μl DNA template (1 ng/μl) covered with 20 μl mineral oil: distribution of the mixtures and mineral oil was performed by an automatic BIOMEC 2000 robot. Amplification was performed in a PT100 thermocycler (MJ Research) using the step-down protocol (Hecker and Roux, 1996) modified for amplification of *T. cruzi* DNA: initial denaturation step at 94 °C for 5 min, annealing at 58 °C for 30 s; extension at 72 °C for 30 s and denaturation step at 94 °C for 30 s. At each five cycles, the temperature of annealing was decreased by 2 °C down to 48°C. At this temperature the number of cycles increased to 10, followed by a final extension step at 72 °C for 7 min. The microsatellite amplicons were loaded on a 6% denaturing polyacrylamide gel and analyzed in an ALF sequencer (Pharmacia) using the Allelinks software.

2.5. Phylogenetic analysis

To estimate the genetic divergence among the stocks, Jaccard's genetic dissimilarity index was used (Jaccard, 1908). It measures the proportion of band mismatches between a pair of stocks according to the following formula: $D = 1 - (a/(a + b + c))$, where a = number of bands that are common to the two compared genotypes, b = number of bands present in the first genotype and absent in the second and c = number of bands absent in the first genotype and present in the second. The unweighted pair group method with arithmetic averages method (UPGMA) (Sneath and Sokal, 1973) was used to construct phylogenetic trees based on these distances. Different softwares were

used: "Genetics" (unpublished) to compute distances, "Neighbor" from the package PHYLIP (Felsenstein, 1989) to manage UPGMA protocols and "Treeview" to display the trees (Page, 1996).

3. Results

3.1. MLEE patterns of Be-62 and Be-78 strains

We analyzed 22 enzymatic loci of the Be-62 and Be-78 *T. cruzi* strains (Table 1). From these, only DIA-NADH and PGM enzymes showed slight differences between both strains. Be-62 and Be-78 strains were very similar to each other and also with clone MAScl1, used as a reference genotype. Both differed from MAScl1 in relation to DIA-NADH, but only Be-78 strain differed from MAScl1 in relation to PGM. Fig. 1 shows a representative result obtained with PGM.

3.2. RAPD patterns of Be-62 and Be-78 strains

Nine primers were used in an attempt to differentiate the Be-62 and Be-78 strains. Only three out of tested

Table 1
Comparative MLLE profiles between Be-62 of and Be-78 *Trypanosoma cruzi* strains

Enzymes	*T. cruzi* strains and MLEE profiles	
	Be-62	Be-78
GPI	3	3
MDH	6	6
ME1	3	3
ME2	4	4
DIANADH	**3**	**5**
DIANADPH	4	4
6PG	6–7	6–7
PEP1	3–5	3–5
PEP2	2	2
Nhi	1	1
GCH1	5	5
GDH2	4	4
GOT	4	4
MPI	1	1
GAPD	6	6
PGM	**9–11**	**8–11**
ALAT	3	3
LAP	4	4
G6PD	2	2
IDH	5–6	5–6
SOD	6–7	6–7
ACON	9–9	9–9

Numbers indicate the relative position of bands in the electrophoresis gels. Bold faces indicate the enzymes with different electrophoresis mobility between Be-68 and Be-72.

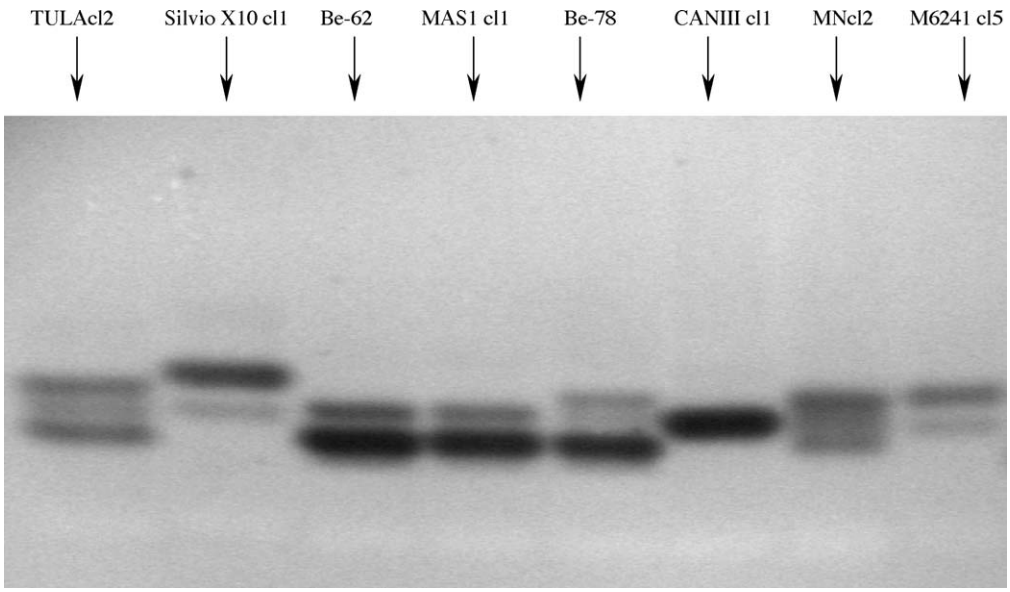

Fig. 1. Isoenzyme patterns of PGM are showing differences between the Be-62 and Be-78 strains and similarity of both with the clone MAScl1 belonging to the genotype 32 of *Trypanosoma cruzi*. The sequence of the stocks assayed was: TULAcl2 (genotype 43), SilvioX10cl1 (genotype 19 and 20), Be-62 strain, MAS1cl1 (genotype 32), Be-78 strain, CANIIIcl1 (genotype 27), MNcl2 (genotype 39) and M6241cl5 (genotype 36).

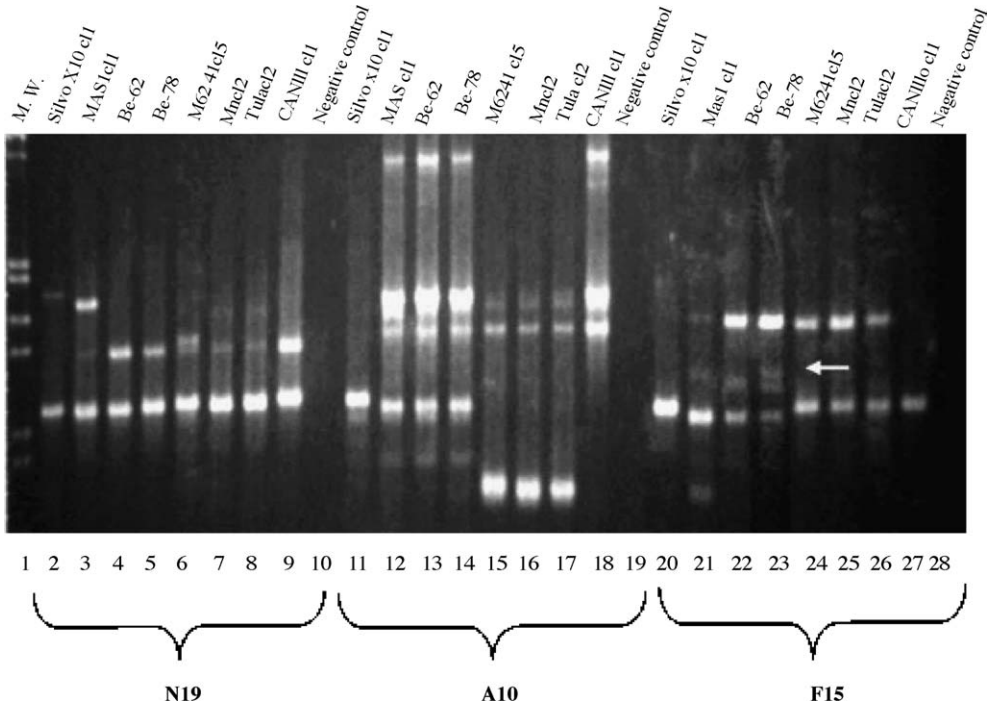

Fig. 2. Representative RAPD patterns of the Be-62 and Be-78 strains and *T. cruzi* from different genotypes. Lane 1: 1 kb DNA ladder; lanes 2–10: primer N19; lanes 11–19: primer A10; lanes 20–28: primer F15. For each primer the sequence of the stocks assayed was: SilvioX10cl1 (genotype 19 and 20), MAS1cl1 (genotype 32), Be-62 strain, Be-78 strain, M6241cl5 (genotype 36), MNcl2 (genotype 39), TULAcl2 (genotype 43), CANIIIcl1 (genotype 27) and no DNA template. The white arrow points out the differentiating band between Be-62 strain and Be-78 strain for the primer F15.

primers (F15, N9 and A15) yield products that were different between the two strains. Fig. 2 illustrates one of these differences obtained with the F15 primer. Be-62 RAPD profiles differed from MAScl1 profiles in relation to N19, A10, F15, N9, A15 and B19 loci. Be-78 profiles were also different from those of MAScl1 with the same primers, except for A15 (data not completely shown).

Table 2
Alleles (amplicons in pb) detected for the TcAAT-01, TcTAC-02, TcTAT-01, TcAAAT-01, MCLG10, SCLE10 and MCLE01 microsatellites loci in Be-62 of and Be-78 *Trypanosoma cruzi* strains and their respective clones

Strains and clones	Loci						
	TcAAT-01	TcTAC-02	TcTAT-01	TcAAAT-01	MCLG10	SCLE10	MCLE01
Be-62	**251–259**	**257–259**	**183–201**	**256–271**	**175–177**	**273–275**	**257–274**
Be-62cl3	251–259	257–259	183–201	256–271	175–177	273–275	257–254
Be-62cl8	251–259	257–259	183–201	256–271	175–177	273–275	257–254
Be-62cl7	251–259	257–259	183–201	256–271	175–177	273–275	257–254
Be-62cl12	251–259	257–259	183–201	256–271	175–177	273–275	257–274
Be-78	**262–270**	**262–264**	**197–207**	**272–275**	**177**	**268**	**149–157**
Be-78cl2	262–270	262–264	197–207	272–275	177	268	149–157
Be-78cl9	262–270	262–264	197–207	272–275	177	268	149–157
Be-78cl10	262–270	262–264	197–207	272–275	177	268	149–157
Be-78cl15	262–270	262–264	197–207	272–275	177	268	149–157

Data in bold refer to parental *Trypanosoma cruzi* strains.

3.3. Microsatellite patterns of the parental strains Be-62 and Be-78 and their clones

We profiled the nuclear genome of both parental strains and their respective clones by analyzing seven microsatellite loci (Table 2). For all investigated loci the fluorescent PCR products were different between the strains Be-62 and Be-78. However, the microsatellite profiles of the clones were equivalent to their respective parental strains, indicating that both strains are

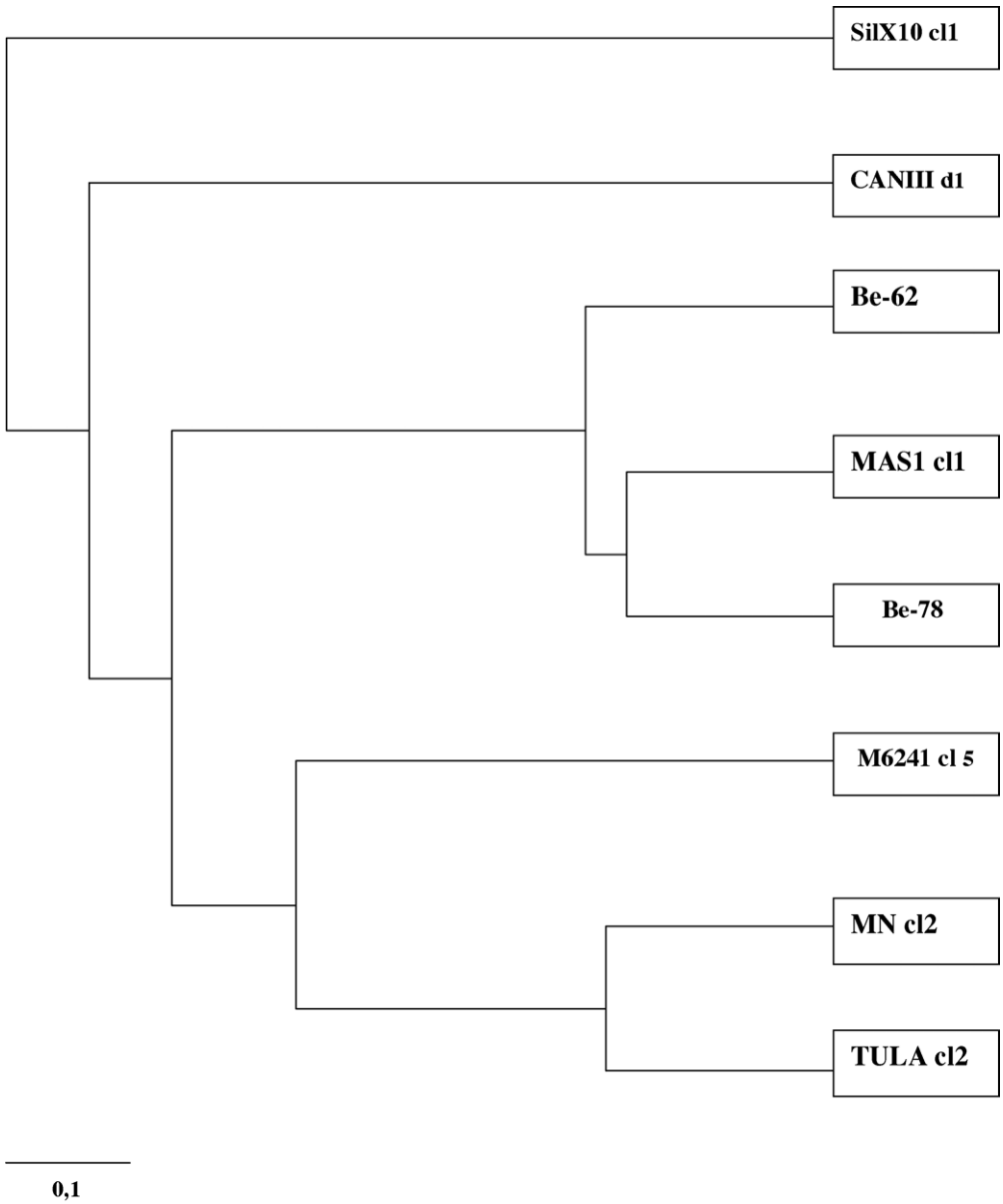

Fig. 3. An unweighted pair-group method with arithmetic averages (UPGMA) dendogram depicting the phylogenetic relationships among Be-62 and Be-78 strains and clones belonging to the principal genetic groups of *T. cruzi* (Tibayrenc and Ayala, 1988), assayed by isoenzymatic electrophoresis and RAPD. The scale indicates genetic distances estimated with the index of Jaccard (1908).

probably monoclonal populations. This hypothesis is corroborated by the fact that multi-peak patterns were not observed for the parental strains. We detected only one-peak or two-peak profiles, interpreted as homozygosis and heterozygosis at the specific locus for both Be-62 and Be-78 strains.

3.4. Phylogenetic analysis

For the phylogenetic analyses we combined the isoenzyme and RAPD data and constructed an UPGMA dendogram showed in Fig. 3. As expected, Be-62 and Be-78 were much more similar to MAScl1 than all the other reference clones used.

4. Discussion

The Be-62 and Be-78 strains, although isolated from the same patient in 1962 and in 1978, respectively (Salgado et al., 1962; Lana and Chiari, 1986), display some important biological and molecular differences (Lana and Chiari, 1986; Lana et al., 1996, 1992; Araújo et al., 2002.). These findings raised questions about the differences in the two populations and their phylogenetic relationship. In addition, we were interested to know whether Berenice patient was infected with a multiclonal *T. cruzi* population and if we could identify this supposed populations mixture in the two isolated strains. In order to clarify these points we extend the characterization of the Berenice strains and their clones, in the present work, by analyzing 22 enzymatic, 9 RAPD and 7 microsatellite loci.

The comparative isoenzymatic characterization, using the six enzymes previously tested (Lana et al., 1996), and another 16 new systems, showed differences in only two loci: PGM, partially confirming previous results of Lana et al. (1996) and DIA-NADH. The similarity of MDH profile now observed may be consequence of the parasites manipulation in the laboratory by successive blood passage in mice as demonstrated by Carneiro et al., (1991). Both strains were very similar to the MAScl1 clone of *T. cruzi*, used as reference for the genotype 32 (Tibayrenc and Ayala, 1988).

RAPD data showed slight differences in three out of nine primers. Both strains were also related to the genotype 32 of Tibayrenc and Ayala (1988) corroborating the results described above. The UPGMA dendogram built from the MLEE and RAPD combined data confirmed this notion.

To verify if the two Berenice strains are mono or multiclonal populations, both *T. cruzi* strains were cloned, and their clones comparatively analyzed by the microsatellite technique. Considering the sensitivity of this technique the results obtained also showed differences between the parental strains; however, they suggested that both strains are probably monoclonal populations since each strain and their respective clones showed the same amplification profiles with only one or two peaks in all seven loci tested (Oliveira et al., 1998).

Taking together, our data suggest that although the Be-62 and Be-78 strains were different in several important biological properties these strains are in fact phylogenetically very closely related. Both strains can be considered as belonging to the genetic group 32 of Tibayrenc and Ayala (1988), equivalent to Zymodeme A of Romanha et al. (1979) or Z2 of Miles et al. (1978) or Lineage I of Souto et al. (1996) or *T. cruzi* II group (Satellite Meeting, Rio de Janeiro, Anon, 1999) which, at least in Brazil, comprises *T. cruzi* that are predominant in the domestic cycle of the disease.

Finally, although we have confirmed results previously reported (Lana et al., 1996) demonstrating that Be-62 and Be-78 are different populations, we were not able to detect the presence of subpopulations in the two strains with the parameters used here, suggesting that more discriminating and sensitive molecular markers may still be necessary for a better characterization of these strains. These results do not allow us to discard the possibility that the patient Berenice was infected more than once during her life. However, if we admit that Berenice had been infected only once, the host immune system might have played an important role in the modulation of *T. cruzi* population during the long-term infection.

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