Relationship between Biological Behaviour and Randomly Amplified Polymorphic DNA Profiles of *Trypanosoma cruzi* Strains

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Once known some biological characteristics of six Trypanosoma cruzi *strains, randomly amplified polymorphic DNA (RAPD) analysis was made. Cluster analysis by UPGMA (unweighted pair group method analysis) was then applied both to biological parameters and RAPD profiles. Inspection of the UPGMA phenograms indicates identical clusters, so supporting that usefulness of biological parameters to characterization of* T. cruzi *strains still remains.*

Key words: *Trypanosoma cruzi* - strains - characterization - randomly amplified polymorphic DNA (RAPD)

Attempts to correlate biological, biochemical and genetic characteristics of *Trypanosoma cruzi* isolates with the clinical variability of Chagas disease have been reported (Gonzalez et al. 1995, Montamat et al. 1996). Studies of natural populations of the parasite have revealed interesting data. Tibayrenc et al. (1986), Tibayrenc and Ayala (1987) and Laurent et al. (1996) suggest a clonal structure in *T. cruzi* by isoenzymes analysis and kDNA sequences. Miles et al. (1980), Bogliolo et al. (1986) and Carneiro et al. (1991) define three zymodemes in several surveys performed in Brazil by using immunoenzymatic markers. Steindel et al. (1993) suggest the existence of a great number of individual genotypes into the species, based on the diversity of randomly amplified polymorphic DNA (RAPD) profiles. Bogliolo et al. (1996) even report evidences of genetic recombination from polymorphisms in *T. cruzi*. The presence of principal clones in some geographic areas, showing a high percentual of similarity by restriction fragment length polymorphism (RFLP) analysis (Campos et al. 1999) could also contribute to the pattern of clinico-pathological manifestations of Chagas disease.

The present work attempts to correlate the heterogeneity of the biological characteristics shown by several strains of the parasite with their RAPD profiles. For this purpose, unweighted pair groups method analysis (UPGMA) phenograms are built both with biological and genetic data.

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MATERIALS AND METHODS

Parasites and DNA preparation - Four strains of *T. cruzi* were used to DNA extraction: Bolivia strain (Funayama & Prado Junior 1974), RAL strain (Ribeiro et al. 1993), GM strain (Prado Junior et al. 1992a) and MC strain (Prado Junior et al. 1992b). After infecting groups of NMRI mice by the intraperitoneal route, samples of blood obtained by cardiac puncture were cultured in glass tubes with 5 ml LIT medium (10% heat-inactivated fetal calf serum) and two subcultures were made before beginning the assays.

Biological characteristics of these four strains, as well as Y strain have been previously determined and a summary is indicated in Table I. As can be seen, some parameters for Tulahuen strain (Segura et al. 1980), as growth of epimastigotes in LIT and susceptibility to drugs (data not published), have been included.

To DNA preparation, parasites were cultured for 7-9 days in LIT medium at 28ºC using 80 ml glass flasks. Cultures were centrifuged at 900 g 10 min at 4°C and washed three times in PBS (phosphate buffered saline), pH 7.4. Pellet of parasites was resuspended in 50 mM Tris-HCl/ 50 mM EDTA/100 mM NaCl/0.5% SDS, pH 7.6 and incubated together with 20 mg/ml proteinase K for 2 h at 55°C. Following two phenol extractions and 3M ethanol-acetic acid precipitation, DNA was resuspended in 10 mM Tris-HCl/1 mM EDTA pH 8.0 (TE). Valuation was made in spectrophotometer at 260-280 nm to determine the purity rate.

Promastigotes of cultures of *Leishmania infantum* and larvae of *Trichinella spiralis* isolated by digestion were processed as above to extraction of DNA.

PCR amplification and polyacrilamide gel electrophoresis - Each amplification reaction was done in a final volume of 10 µl containing 0.5 units of *Replitherm* DNA polymerase (Epicentre Technologies)/ 2.5 mM of each dNTP/ 200 mM Tris-ClH, pH 8.3/ 1M ClK/ 0.1% Tween 20/ 0.1% NP-40/ 25 mM Cl_2Mg together with 0.01 µg of template DNA. The primers B1 (5´-CTTTCGCTCC-3´), B2 (5´-CTGCTGGGAC-3´) and Z3 (5´-CAGCACC GCA-3´), from Operon Technologies, were arbitrarily selected. Amplification was made in Genetic Thermal Cycler GTC-2 (Precision Scientific) with an initial denaturation at 95°C for 5 min, following two cycles at 30°C for 2 min for annealing, 72°C for 1 min for extension and 30 sec at 95°C for denaturation; next, 33 cycles at 40°C for 2 min, 72°C for 1 min and 30 sec at 95°C. In the final cycle the extension step was for 5 min (Steindel et al. 1993). Amplified DNA samples were subjected to electrophoresis through a 4% polyacrilamide gel. Gels were fixed with 10% ethanol/ 0.5% acetic acid for 5 min, stained with 0.2% silver nitrate for 10-20 min and reduced with NaOH and formaldehyde until appearance of bands.

TABLE I

Some biological characteristics of studied strains

	Bolivia strain	RAL strain	GM strain	MC strain	Y strain	Tulahuen strain
Biological source	*Triatoma infestans*	*Triatoma infestans*	*Felis yagouaroundi*	*Chrysocion brachiurus*	Human	*Triatoma*
Geographical source	Vitichi (Bolivia)	São Paulo (Brazil)	Mato Grosso (Brazil)	Mato Grosso (Brazil)	São Paulo (Brazil)	Tulahuen (Chile)
Mortality rate in mice	83.4%		100%	93.3%	100%	100%
Peak of parasitaemia	49,000 try/ml		39,000 try/ml	28,000 try/ml	10,000 try/ml	
Time of highest parasitaemia	day 15th p.i.	day 12th p.i.	day 10th p.i.	d. 7-16th p.i	day 11th p.i.	day 14th pi
Peak of growth in LIT medium	27,300,000	20,850,000	22,500,000	23,900,000	26,450,000	24,375,000
Time of highest growth in LIT medium	day 11th	day 11th	day 16th	day 9th	day 11th	day 11th
Highest % of metacyclic forms in Grace medium	33%	13.8%	46.5%	8.3%	14%	
Time of highest % of metacyclic forms in Grace medium	day 9th	day 11th	day 11th	day 14	day 9th	
Infection ability in fibroblasts	29.7%	16.4%	28.6%	15%	16.7%	
Infection ability in macrophages	25.9%	26.2%	44.1%	12.5%	14.7%	
Length of intracellular cycle in fibroblasts	6 days	6 days	6 days	6 days	6 days	
Length of intracellular cycle in macrophages	6 days	>6 days	6 days	6 days	6 days	
Susceptibility to nifurtimox (reduction % to 100, 10 and 1 µg/ml)	99.2 90.9 0	100 82.5 0	98.5 89.5 0	99.1 87.9 0	100 92.9 0	98.1 96.2 0
Susceptibility to benznidazole (reduction % to 100, 10 and 1 µg/ml)	84.7 0 0	100 0 0	81.8 0 0	97.2 0 0	100 48.3 0	97.9 75.5 0
Susceptibility to gentian violet (reduction % to 100, 10 and 1 µg/ml)	100 71.8 0	100 75.4 0	100 67.2 0	100 88.2 0	100 82.4 75.8	100 75.5 61.2

Data analysis - Data obtained both from biological parameters and RAPD profiles were subjected to the same statistical procedure. NTSYS (Applied Biostatistics Inc., Setauket, NY) informatic program was used. This program allows the establishment of possible strain groups based on the similarities and differences observed. Briefly, we elaborated matrices of similarity assigning 0 or 1 values to the results of the assays. These matrices were processed using the Simple Matching coefficient (SM; Sneath & Sokal 1973) and phenetic trees were plotted using the UPGMA. Although artificial, UPGMA dendogram constructed from biological parameters, makes comparison of results easier.

RESULTS

Criteria from biological parameters used for performing the matrix of similarity are shown in Table II. By the Simple Matching coefficient, the matrix of similarity (Table III) was used to cluster analysis, resulting the UPGMA phenogram indicated in Fig. 1.

Regarding to RAPD profiles, 0 or 1 values of

TABLE II

Criteria employed to the construction of the behaviour matrix

Mortality rate in mice	$<100\% \rightarrow 0$ $100\% \rightarrow 1$	
Peak of parasitaemia	low parasitaemia high parasitaemia	$(10^4/ml) \rightarrow 0$ $(> 2x10^4/ml) \rightarrow 1$
Time of highest parasitaemia	≤ 12 days $\rightarrow 0$ >12 days $\rightarrow 1$	
Peak of growth in LIT medium	$<25x10^6 \rightarrow 0$ $>25x10^6 \rightarrow 1$	
Time of highest growth in LIT medium	$\leq$day 11th $\rightarrow 0$ $>$day 11th $\rightarrow 1$	
Highest % of metacyclic forms in Grace medium	$\leq 2x8,3 \rightarrow 0$ $>2x8,3 \rightarrow 1$	(8,3: less value)
Total number of metacyclic forms in Grace culture peak	$\leq 10x10^6 \rightarrow 0$ $>10x10^6 \rightarrow 1$	
Time of highest % of metacyclic forms in Grace medium	≤ 11 days $\rightarrow 0$ >11 days $\rightarrow 1$	
Infection ability in fibroblasts (% of infected cells 24 h p.i.)	$\leq 17\% \rightarrow 0$ $>17\% \rightarrow 1$	$(\leq 1/2\%max \rightarrow 0)$ $(> 1/2\%max \rightarrow 1)$
Infection ability in macrophages (% of infected cell 24 h p.i.)	$\leq 17\% \rightarrow 0$ $>17\% \rightarrow 1$	$(> 1/2\%max \rightarrow 0)$ $(> 1/2\%max \rightarrow 1)$
Length of intracellular cycle in fibroblasts	<6 days $\rightarrow 0$ >6 days $\rightarrow 1$	
Length of intracellular cycle in macrophages	6 days $\rightarrow 0$ >6 days $\rightarrow 1$	
Susceptibility to nifurtimox (reduction % at 100 µg/ml)	$<100\% \rightarrow 0$ $100\% \rightarrow 1$	
Susceptibility to nifurtimox (less concentration with trypanocidal activity)	≤ 10 µg/ml $\rightarrow 0$ >10 µg/ml $\rightarrow 1$	
Suscetibility to benznidazole (reduction % at 100 µg/ml)	$<100\% \rightarrow 0$ $100\% \rightarrow 1$	
Susceptibility to benznidazole (less concentration with trypanocidal activity)	≤ 10 µg/ml $\rightarrow 0$ >10 µg/ml $\rightarrow 1$	
Susceptibility to gentian violet (reduction percentage at 100 µg/ml)	$<100\% \rightarrow 0$ $100\% \rightarrow 1$	
Susceptibility to gentian violet (less concentration with trypanocidal activity)	≤ 1 µg/ml $\rightarrow 0$ >1 µg/ml $\rightarrow 1$	

TABLE III

Matrix of simple matching coefficients for phenogram of Figure 1

	Bolivia strain	RAL strain	GM strain	MC strain	Y strain	Tulahuen strain
Bolivia strain	1,000					
RAL strain	0,556	1,000				
GM strain	0,722	0,500	1,000			
MC strain	0,667	0,667	0,611	1,000		
Y strain	0,444	0,667	0,389	0,556	1,000	
Tulahuen strain	0,556	0,556	0,500	0,667	0,778	1,000

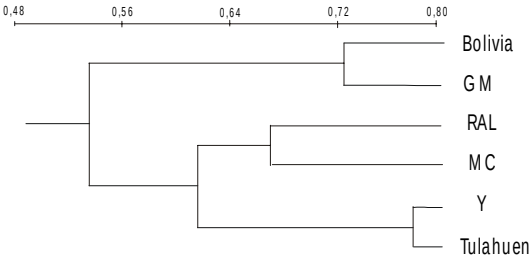

Fig. 1: unweighted pair group method analysis phenogram of *Trypanosoma cruzi* strains based on biological characteristics and behaviour. The similarity coefficient shown on the horizontal scale was derived from the Simple Matching coefficient.

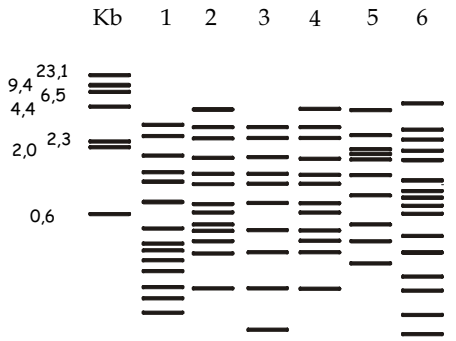

Fig. 2: randomly amplified polymorphhic DNA profile of *Trypanosoma cruzi* strains using the primer Z3. Lines: 1 - Bolivia strain; 2 - RAL strain; 3 - GM strain; 4 - MC strain; 5 - *Leishmania infantum* sample; 6 - *Trichinella spiralis* sample. The line on the left shows molecular size markers.

the matrix are based in the presence or absence of bands in the electrophoretic patterns (Figs 2, 3, 4, respectively). As above, the resulting matrix of similarity coefficients is shown in Table IV and the corresponding UPGMA phenogram is shown in Fig. 5.

DISCUSSION

The extent of heterogeneity of *T. cruzi* isolates has been widely demonstrated from biological and genetic studies. Early isoenzymatic analysis or studies with restriction endonucleases have allowed the establishment of groups of *T. cruzi* strains according to different characteristics as geographical source or pathogenicity (Miles et al. 1978, 1980, Tibayrenc & Desjeux 1984). *T. cruzi* taxon is presently subdivided into two major phylogenetic lineages (Tibayrenc 1995, Souto et al. 1996), each retaining considerable heterogeneity. Zymodeme Z1 stocks belong to lineage 2, which cycles in a sylvatic environment but has the capacity to colonize human settlements, whereas zymodeme Z3, which also cycles in a sylvatic environment, is clustered into lineage 1 (Tibayrenc 1995, Souto et al. 1996). Zingales et al. (1998) found strong association of lineage 1 with the domestic cycle, though in the sylvatic cycle both lineages circulate equally.

In this paper, diversity of individual genotypes by RAPD analysis and its relation to the previously determined biological behaviour is approached. For this purpose, cluster analysis using the UPGMA

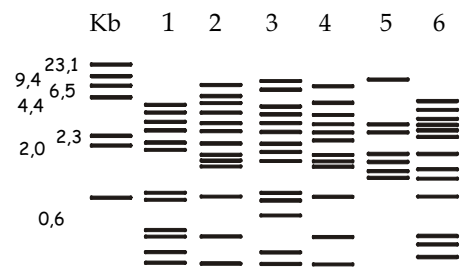

Fig. 3: randomly amplified polymorphhic DNA pattern of *Trypanosoma cruzi* strains using the primer B10. Lines: 1 - Bolivia strain; 2 - RAL strain; 3 - GM strain; 4 - MC strain; 5 - *Leishmania infantum* sample; 6 - *Trichinella spiralis* sample. The line on the left shows molecular size markers.

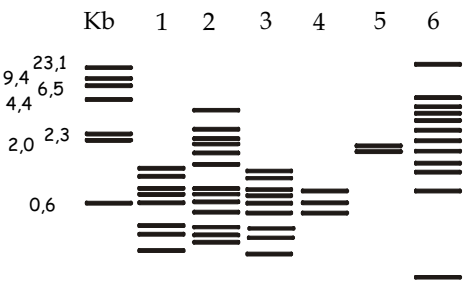

Fig. 4: randomly amplified polymorphhic DNA profile of *Trypanosoma cruzi* strains using the primer B1. Lines: 1 - Bolivia strain; 2 - RAL strain; 3 - GM strain; 4 - MC strain; 5 - *Leishmania infantum* sample; 6 - *Trichinella spiralis* sample. The line on the left shows molecular size markers.

TABLE IV

Matrix of simple matching coefficients for phenogram of Figure 5

	Bolivia strain	RAL strain	GM strain	MC strain	*Leishmania infantum*	*Trichinella spiralis*
Bolivia strain	1,000					
RAL strain	0,652	1,000				
GM strain	0,888	0,674	1,000			
MC strain	0,697	0,843	0,719	1,000		
L. infantum	0,551	0,584	0,573	0,674	1,000	
T. spiralis	0,427	0,303	0,427	0,438	0,449	1,000

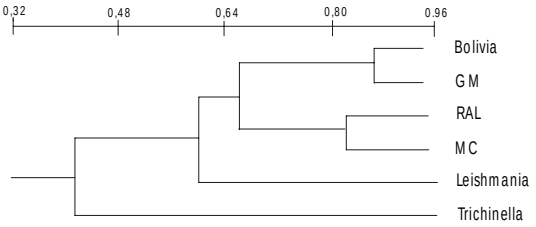

Fig. 5: unweighted pair group method analysis phenogram of *Trypanosoma cruzi* strains based on pairwise band sharing from randomly amplified polymorphic DNA profiles. The similarity coefficient shown on the horizontal scale was derived from the Simple Matching coefficient. This tree includes samples from *Leishmania infantum* promastigotes and *Trichinella spiralis* larvae processed in the same conditions that *Trypanoasoma cruzi* samples.

was performed. A phenogram based on band sharing was constructed to show genetic relationships among the strains studied. By RAPD analysis and isoenzyme studies, Steindel et al. (1993) and Lopez-Olmos et al. (1998) have found similarities among isolates from the same geographical source. In our case, the variability found is not related to factors previously known as geographical or biological source or pathogenicity.

Because of the number of strains characterized, results obtained must be considered as preliminary data; however, an interesting and unexpected finding is the same association among strains, both by their biological characteristics and their RAPD profiles. So, the phenogram constructed from biological data revealed that there are two principal lines; the first one clusters Bolivia and GM strains, and the second one shows two groups of pairs: RAL/MC and Y/Tulahuen.

When RAPD fingerprintings were used in a cluster analysis of combined electrophoretic patterns, the phenogram allowed the separation of two groups based on their divergence: *Trichinella* and the protozoa. The latter shows clear differences among *T. cruzi* strains and the reference sample from *Leishmania.* These results emphasize the usefulness of RAPD to the study of parasite populations. According to Steindel et al. (1993), the technique is ideal for searching genetic markers for the various biological characteristics of the parasite and the clinical variations in the disease it causes.

As above, phenon line of *T. cruzi* strains shows a new division in two pairs: Bolivia/GM and RAL/MC strains.

After all, the two methods of analysis led us to draw the same conclusion. It seems obvious that the differences in the biological behaviour are related to variations in the genomic constitution expressed by the amplification of DNA fragments. However, having in mind the low number of strains analyzed, it would be necessary to perform new assays with more sensitive techniques, or increase the number of compared strains in a similar study. Furthermore, the establishment of clones from the strains must be the next step in the continuation of this study. After the enlargement of these studies we will be able to check if the two major branches found in this work, both for biological parameters and RAPD profiles, correspond to the two major phylogenetic lineages TC 1 and TC 2.

Determination of the number of amplified DNA fragments in common is a technique extremely simple to study phylogeneytic relationships among organisms. However, the characterization of *T. cruzi* strains by measuring of biological parameters, such as growth kinetics in axenic liquid medium, in vitro metacyclogenesis, parasitemias and mortality rates in mice, infection of cultured cells (Gonzalez et al. 1995, Lauria-Pires et al. 1997, Lopez-Olmos et al. 1998, Solari et al. 1998, Almeida-de-Faria et al. 1999) follow nowadays applying to knowledge of diversity Chagas disease. The correlation found by us between RAPD profiles and biological behaviour of *T. cruzi* strains confirms the importance of the biological analysis, and this is the main aim of this paper.

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