

## Biological Characterization of *Trypanosoma cruzi* Strains

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*Biological parameters of five Trypanosoma cruzi strains from different sources were determined in order to know the laboratory behaviour of natural populations. The parameters evaluated were growth kinetics of epimastigotes, differentiation into metacyclic forms, infectivity in mammalian cells grown in vitro and parasite susceptibility to nifurtimox, benznidazole and gentian violet. Differences in transformation to metacyclic, in the percentage of infected cells as well as in the number of amastigotes per cell were observed among the strains. Regarding to pharmacological assays, Y strain was the most sensitive to the three assayed compounds. These data demonstrate the heterogeneity of natural populations of T. cruzi, the only responsible of infection in humans.*

Key words: *Trypanosoma cruzi* - strains - characterization

*Trypanosoma cruzi* is the etiologic agent of Chagas disease, a frequently fatal illness affecting the heart and gastrointestinal systems. An estimated 16 to 18 million people in Latin America and 50,000 to 100,000 people in the United States are infected with this pathogen (WHO 1991). A striking feature of *T. cruzi* is its heterogeneity in relation to biological properties. Differences in growth rates, infectivity, tissue tropism, antigenic composition, virulence and morbidity in animal models and susceptibility to immune sera and chemotherapeutic drugs have been reported in parasite isolates (Andrade et al. 1975, 1985, Brener et al. 1976, De Castro & De Meirelles 1987, Melo & Brener 1978, Neal & Van Bueren 1988, Roval et al. 1990). In humans, a broad spectrum of clinical presentations in Chagas disease is observed, possibly reflecting the heterogeneity among *T. cruzi* isolates and/or genetic differences in the immune response of the host (Brener 1980).

Early studies also revealed substantial isozymic variability among isolates, defining three major groups or zimodemes (Miles et al. 1980). Characterization of different classes of parasites has been also achieved by studying restriction fragment length polymorphism of kDNA (Morel et al. 1980). Therefore, both biological and genetic char-

acteristics have shown a marked polymorphism in natural populations of *T. cruzi*.

Nowadays, the advances in the fields of molecular biology and genetic are allowing to elucidate some of the reasons for such a variability in the biological behaviour of *T. cruzi* strains. So, Allaoui et al. (1999) have applied genetic manipulations techniques to ascertain the biological functions of *T. cruzi* Tc52 protein. This is a recently identified protein (Ouaissi et al. 1995a) encoded by a single pair of allelic genes, that increases during the stationary growth phase of epimastigotes cultures, with interesting enzymatic and immunosuppressive activities (Ouaissi et al. 1995b, Fernandez-Gómez et al. 1998).

Although the possibility of assays with transformed parasites by gene manipulation is now a reality, the true is that the heterogeneous populations that are maintained in nature are the only responsible of infection in humans and a large number of other mammalian species.

By this reason, the aim of this work is to know biological characteristics of these natural populations of *T. cruzi*, including growth kinetics of epimastigotes, metacyclogenesis in Grace medium, infectivity in mammalian cells grown in vitro and finally, the susceptibility to nifurtimox, benznidazole and gentian violet. These characteristics can be considered as natural heterogeneity markers and may have important implications in the clinic features of Chagas disease.

### MATERIALS AND METHODS

*T. cruzi* strains - Five strains of *T. cruzi* from different biological and geographic origins were

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studied. Bolivia strain (Funayama & Prado Junior 1974) was isolated from *Triatoma infestans* in Vitichi (Bolivia) and it causes high parasitaemias and moderate mortality in mice. RAL strain (Ribeiro et al. 1993) was also isolated from *T. infestans* in São Paulo (Brazil). GM strain (Prado Junior et al. 1992a) from Brazil was isolated by xenodiagnostic from *Felis yagouaroundi* coming from Mato Grosso; this strain causes high parasitaemia and mortality in mice. MC strain (Prado Junior et al. 1992b) was isolated in Brazil from *Chrysocyon brachyurus*, and it reaches moderate parasitaemia and mortality in mice. Finally, Y strain (Silva & Nussenzweig 1953) was isolated from an acute human case coming from Marília (São Paulo, Brazil) in 1950; this strain is responsible of low parasitaemia but high mortality in mice.

Groups of NMRI mice were inoculated with every strain. After exsanguination, blood of infected mice was cultured in glass tubes with 5 ml LIT medium (10% heat-inactivated fetal calf serum) and two subcultures were performed in this medium before beginning the assays.

**Growth curves** - To determine the growth rates of the parasite strains,  $5 \times 10^5$  epimastigotes/ml were seeded in LIT medium supplemented with 10% FCS and maintained at 28°C. Number of epimastigotes were assessed in a hemocytometer.

**Metacyclogenesis** - Metacyclogenesis was achieved in Grace medium (Grace 1962) supplemented with 10% FCS. Supernatants from LIT axenic cultures, containing live forms, were collected after centrifugation and then washed with fresh LIT medium. Nine millions of trypanosomes/ml were seeded in glass tubes with 5ml Grace medium. Metacyclic trypomastigotes were observed from 48 h onwards. Aliquots of the supernatants were fixed and stained with Giemsa to follow the kinetics of differentiation into metacyclic forms.

**Infectivity to cultured mammalian cells** - Two cell lines were used: Vero fibroblasts, maintained in Minimal Essential Medium (20% FCS) and J774 macrophages maintained in RPMI medium (20% FCS). In both cases 15,000 cells/well were seeded on sterile round coverslips placed in 24-well plates. Then 150,000 trypomastigotes from Grace medium were added to each well, giving a final volume of 2 ml. Metacyclic trypomastigotes were allowed for 24 h to invade cells; culture medium was removed and fresh medium were added. Infected cells were incubated at 37°C in 5% CO<sub>2</sub>. Cells grown on coverslips were fixed with methanol and stained with Giemsa stain for microscopic counts of number of infected cells and number of amastigotes per infected cell.

**Susceptibility to chemotherapeutic agents** - Parasite sensitivity to nifurtimox, benznidazole and gentian violet was assayed on epimastigote cultures as previously described (Herrero et al. 1992). Five concentrations ranging from 100 to 0.01 µg/ml were used. Growth indexes, growth percentages and reduction percentages respect to control untreated cultures were calculated as follows:

$$\text{Growth index (G.I.)} = \frac{\text{Final number of parasites}}{\text{Initial number of parasites}}$$

Growth percentage (G.P.). Only when G.I. experimental group > 1

$$\text{G.P.} = \frac{\text{G.I. experimental group}}{\text{G.I. control group}} \times 100$$

Reduction percentage (R.P.). Only when G.I. experimental group < 1

$$\text{R.P.} = 100 - \left( \frac{\text{G.I. experimental group}}{\text{G.I. control group}} \right) \times 100$$

## RESULTS

The growth rates of the parasite strains measured for 22 days of culture were not different (Fig. 1). All strains achieved a growth peak over 20 millions epimastigotes/ml between days 9th and 11th of culture, with the only exception of GM strain, that delayed its peak at day 16th. The stationary phases were around 15-20 millions of epimastigotes/ml for Bolivia, GM, MC and Y strains, while RAL strain only reached 10 millions.

The transformation of *T. cruzi* epimastigotes to the mammalian infective metacyclic trypomastigotes showed more variability (Fig. 2), ranging from counts of 10 (GM) to 3 (RAL, MC and Y) millions of trypomastigotes/ml. In all cases, the higher levels of trypomastigotes were found after day 9th (Table II).

Assays of infectivity to Vero cells and J774 macrophages revealed differences for both RAL and GM strains (Fig. 3). Also, as can be seen in Figs 4 and 5, all the strains, with the only exception of RAL, completed their intracellular multiplication in six days, releasing the second generation of trypomastigotes responsables of new infections in healthy cells.

Finally, differences in sensitivity to nifurtimox, benznidazole and gentian violet could be appreciate among strains (Table I).

## DISCUSSION

Chagas disease has a broad variety of clinical presentations that may be the result either of heterogeneity among the *T. cruzi* isolates or of the host immune response (Brener 1980). Regarding

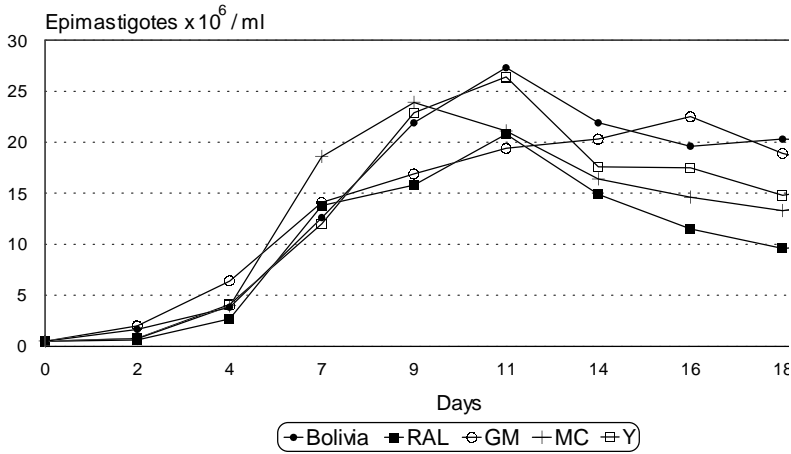


Fig. 1: in vitro growth curves of *Trypanosoma cruzi* in LIT medium.

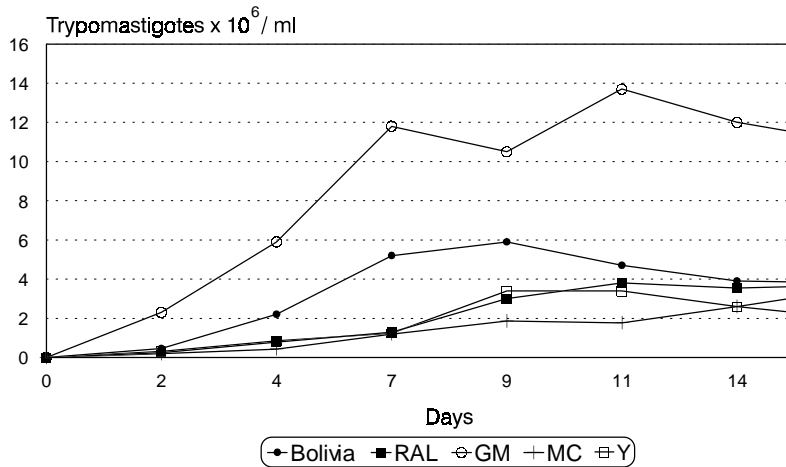


Fig. 2: kinetics of morphological transformation of *Trypanosoma cruzi*. Number of trypomastigotes was determined by differential counting of Giemsa stained smears.

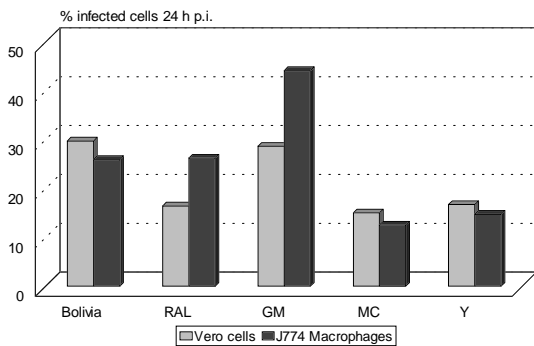


Fig. 3: infection of culture-derived metacyclic trypomastigotes to cell cultures.

to parasite, the infecting strain is a substantial determinant of the evolution of the disease (Montamat et al. 1999).

The diversity in the clinical expression of infection has stimulated interest in studying biological, biochemical and genetic differences among strains. Analysis of natural populations of *T. cruzi* using either isoenzymes or kDNA sequences as genetic markers has revealed a basic clonal structure (Tibayrenc et al. 1986, Tibayrenc & Ayala 1988). Miles and colleagues (Miles et al. 1978, Ready & Miles 1980) defined three zymodemes termed Z1, Z2 and Z3, based on the analysis of stocks from the north and northeast of Brazil. When

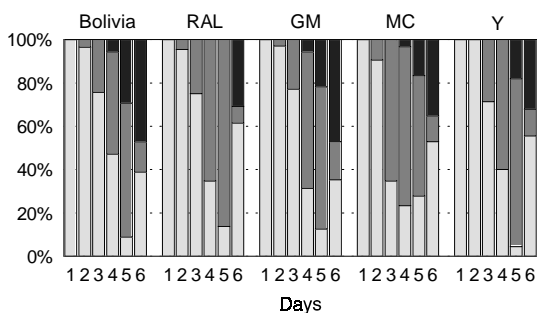


Fig. 4: rate of Vero cells infected with less than five amastigotes of *Trypanosoma cruzi* (□), 5-50 amastigotes (▒) and more than 50 amastigotes (■).

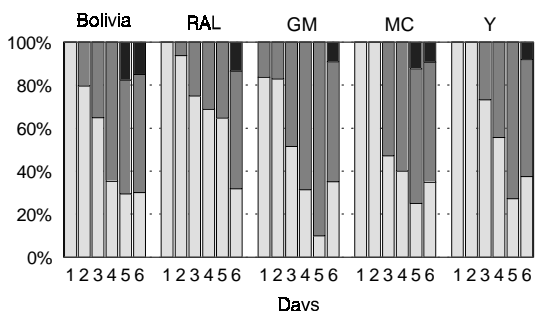


Fig. 5: rate of J774 macrophages infected with less than five amastigotes of *Trypanosoma cruzi* (□), 5-50 amastigotes (▒) and more than 50 amastigotes (■).

random amplification of polymorphic DNA (RAPD) is applied to the study of parasite populations, Steindel et al. (1993) concluded that there is significant genetic variation within zymodemes. Furthermore, different groups of investigators (Carreño et al. 1987, Solari et al. 1992, Tibayrenc & Ayala 1987, Montamat et al. 1999) have demonstrated a close relation between zymodemes and schizodemes, suggesting that nuclear structural genes coding for enzymes and extranuclear DNA (kDNA) have had a parallel evolution.

Because of such variability, the main objective of this work was to know the diversity in the behaviour of *T. cruzi* isolates. Several parameters, selected as natural markers of biological variability have been assessed.

The growth of epimastigotes in LIT medium shows similar dynamics. By contrast, the ability of in vitro metacyclogenesis is clearly higher for GM strain epimastigotes that achieved 40% of transformation to trypomastigotes. Seven to nine days was the time required for the other strains to reach the maximum number of trypomastigotes, results according to Osuna-Carrillo et al. (1979).

In order to know if the variability of the natural populations of *T. cruzi* affect to the intracellular development of parasites, 10 motile trypomastigotes per cell (both fibroblast or macrophage) were added to infect mammalian cell cultures. As the proportion of infected cells is directly related to the number of metacyclic forms (Schenkman et al. 1988), we previously established the 10:1 rate between parasites and cells to compare the intrinsic ability of the strains to infect vertebrate cells. Moreover, the host cellular type can influence on parasitic levels (Ley et al. 1988, Schenkman et al. 1988); there are tissues and cell lines specially susceptible to *T. cruzi* infection while others seldom suffer it. So, we have found the major differences for GM and RAL strains; both of them infect better J774 macrophages than Vero fibroblasts.

Since the only replicative forms in *T. cruzi* life cycle in the vertebrate host are intracellular amastigotes, the cell infection ability of a strain could be related to a higher parasitaemia in mice. That is true for GM and Bolivia strains, that show in our experiences high cell infection rates, and both reach high parasitaemias in mice (Prado Junior et al. 1992a, Atienza 1994) with 39,000 and 49,000 trypanosomes/ml blood, respectively. Nevertheless, the behaviour is not related to mortality; so, while GM kills 100% of experimentally infected mice, Bolivia only causes death of the 83% of the animals (Atienza 1994). Virulence seems to be an intrinsic property of each strain (Kagan et al. 1966, Barbosa et al. 1988); in fact, some strains usually maintained in laboratories are characterized as strains with high pathogenicity and low parasitaemias (Y strain) or strains with low pathogenicity and high parasitaemias (Bolivia) (Silva & Nusenzweig 1953, Funayama 1974). In the same way as pathogenicity is not related to parasitaemia, the infectivity to cultured mammalian cells could not be a consequence of the natural pathogenicity of a strain (Atienza 1994). According to previous data (McCabe et al. 1984, Roval et al. 1990), the duration of the complete intracellular cycle is about six days. Only RAL strain lasts more time to complete its development into J774 macrophages.

The resistance of *T. cruzi* to chemotherapeutic drugs varies from 0% to 100% (Filardi & Brener 1984) and it has been related to mitotropic preferences of strains and predominance of broad blood forms, while sensitive strains are mitotropic or reticulotropic, showing predominance of slender forms (Melo & Brener 1978, Andrade et al. 1985, Neal & Van Bueren 1988). Some aspects as variations in absorption and metabolism of drug, incorporation to different infected tissues, synergic intervention of immune system as well as clinic

TABLE I

Susceptibility of *Trypanosoma cruzi* epimastigotes to nifurtimox, benznidazole and gentian violet expressed as reduction percentages (%R) and growth percentages (%G) regarding untreated controls

Compound	Concentration	Bolivia strain		RAL strain		GM strain		MC strain		Y strain	
		%R	%C	%R	%C	%R	%C	%R	%C	%R	%C
Nifurtimox	100 µg/ml	98.2	0	100	0	98.1	0	99.1	0	100	0
	10 µg/ml	90.9	0	82.5	0	89.5	0	87.9	0	9.9	0
	1 µg/ml	0	82.5	0	82.1	0	80.9	0	87.3	0	58.1
	0.1 µg/ml	0	93.4	0	103.6	0	91.8	0	91.9	0	82.3
	0.01 µg/ml	0	100	0	118.6	0	100	0	103.4	0	96.5
Benznidazole	100 µg/ml	84.7	0	100	0	81.8	0	97.2	0	100	0
	10 µg/ml	0	60.7	0	63.2	0	65.4	0	42.2	48.3	0
	1 µg/ml	0	77.4	0	101.3	0	75	0	70.3	0	88.7
	0.1 µg/ml	0	84	0	120.5	0	85.7	0	80	0	122.5
	0.01 µg/ml	0	91.3	0	112.4	0	95.7	0	94.6	0	120.2
Gentian violet	100 µg/ml	100	0	100	0	100	0	100	0	100	0
	10 µg/ml	71.8	0	75.4	0	67.2	0	88.2	0	82.4	0
	1 µg/ml	0	40.4	0	32.4	0	39.8	0	31.9	75.8	0
	0.1 µg/ml	0	97.4	0	5.8	0	92.3	0	56.8	0	71.4
	0.01 µg/ml	0	101.6	0	87	0	120	0	81.4	0	86.8

TABLE II

Metacyclogenesis process of *Trypanosoma cruzi* strains after seeding supernatants from LIT in Grace medium. The numbers of morphological types were determined by differential counting of Giemsa stained smears

Strain		Days						
		2	4	7	9	11	14	16
Bolivia	a	92.5	75	55	53.8	57.8	62	60.3
	b	3.5	14.5	17.5	13.3	14	11	12
	c	4	10.5	27.5	33	28.3	27	27.8
RAL	a	87.25	79.3	72.8	60.5	67.3	74	73.5
	b	10.75	15.5	18.5	27	19	14.5	13.8
	c	2	8.8	8.8	12.5	13.8	11.5	12.8
GM	a	71.5	69.3	51.5	43.8	44	49	41.3
	b	10.75	7.5	13.5	16.3	9.5	14.5	10.5
	c	17.75	23.3	35	40	46.5	36.5	48.3
MC	a	88	89.8	85.8	75.5	77.3	76	87.3
	b	10.25	7.8	10.8	18.3	16.8	15.8	12.8
	c	1.75	2.5	3.5	6.5	6	8.3	7.5
Y	a	83	84.3	78.8	63.5	66.8	76.3	79
	b	13.5	11	15	22.5	21.2	13.3	10
	c	3.5	4.8	6.3	14	11.5	10.5	11

a: % epimastigote forms; b: % intermediate forms; c: % trypomastigote forms

phase of infection or morphological stage can be implicated (De Castro & De Meirelles 1987, Toledo et al. 1990).

However, most authors think that success of the treatment depends mainly on the strain of *T. cruzi* (Brener & Chiari 1967, Habekorn & Gonert 1972, Andrade et al. 1975, Brener et al. 1976, Andrade & Figueira 1977, Filardi & Brener 1990). By this reason, we assessed the susceptibility as-

says directly on the parasite, employing axenic cultures of epimastigote forms in LIT medium. Nifurtimox and benznidazole showed a trypanocide activity of 100% at 100 µg/ml against two strains (Y and RAL). The former one is also the most sensitive to benznidazole and gentian violet. Previous data about in vivo assays with nifurtimox and benznidazole against Y strain (Ribeiro et al. 1988) also reported it as more sensitive (35 and

57% of cure, respectively) than Bolivia strain (0 and 18%). So, Y strain becomes a good candidate for primary screening of new potential tripanocide agents.

After all, the interest of the biological characterization of *T. cruzi* strains by classical procedures remains, because it contributes with interesting data and it is an important help for our understanding of Chagas disease.

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