Weak relation between genetic and biological diversity of natural populations of *Trypanosoma cruzi*

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ABSTRACT. This paper studies the relation between genetic and biological diversity of natural populations of *Trypanosoma cruzi* (in Parana State, Brazil) isolated from humans (H), classified as *T. cruzi* II, and from sylvatic reservoirs (G) and triatomines (T), classified as *T. cruzi* I. Twenty-five biological parameters were evaluated concerning the kinetic of growth and of metacyclogenesis in mediums LIT and M16, to the infectivity to BALB/c mice, and to the susceptibility to benznidazole. The data support the conclusion of a weak relation between genetic distance and biological diversity using strains. The statistical comparison of the means of each parameter, with regard to the different hosts, showed that group *T. cruzi* II (H) was significantly different from group *T. cruzi* I (G and/or T) in 13 of the 25 parameters studied: group H differed from G and T in three parameters, and from G or T in 10 parameters. The major differences found in those parameters were related to the kinetic of growth and of metacyclogenesis. One biological implication from these differences is that the lowest growth of parasite observed in group *T. cruzi* II (H) makes parasitological diagnoses more difficult and provides lower pathogenicity for mice.

Key words: *Trypanosoma cruzi*, genetic diversity, biological diversity

RESUMO. Fraca relação entre diversidade genética e biológica de populações naturais de *Trypanosoma cruzi*. Este trabalho estudou a relação entre diversidade genética e biológica de populações naturais de *Trypanosoma cruzi* do Estado do Parana, isoladas de humanos (H), classificadas como *T. cruzi* II, e de reservatórios silvestres (G), e triatomíneos (T) classificadas como *T. cruzi* I. Foram avaliados 25 parâmetros biológicos relacionados à cinética de crescimento e de metaciclogênese em meios LIT e M16, à infectividade em camundongos BALB/c e à suscetibilidade ao benznidazol. Os achados falam a favor de uma fraca relação entre distância genética e diferenças biológicas utilizando cepas. A comparação estatística entre as médias de cada parâmetro, considerando os diferentes hospedeiros, mostrou que o grupo *T. cruzi* II (H) foi significativamente diferente do grupo *T. cruzi* I (G e/ou T) em 13 dos 25 parâmetros estudados, onde o grupo H diferiu de G e T em 3 parâmetros e de G ou T em 10 parâmetros. As maiores diferenças ocorreram nos parâmetros relacionados à cinética de metaciclogênese e de crescimento. Entre as implicações biológicas destas diferenças, o crescimento mais lento de parasitas do grupo *T. cruzi* II (H) prejudica o diagnóstico parasitológico e proporciona menor patogenicidade para camundongos.

Palavras-chave: *Trypanosoma cruzi*, diversidade genética, diversidade biológica.

Introduction

Trypanosoma cruzi circulates in nature among humans, vectors and sylvatic and domestic reservoirs. Although the distribution in vectors and reservoirs is much greater than the human infection, it is estimated that 15 million people have this parasite in the Americas (WHO, 2002). In Brazil, there are around 3.5 million people infected (Dias, 1997). In the state of Parana, the infection is endemic, with a prevalence of 4% (Camargo *et al.*, 1984).

After crossing an acute phase line, the patient enters the chronic phase. This chronic phase presents a clinical course that can vary from the asymptomatic

form to the development of serious clinical cardiac and/or digestive forms (Dias, 1992). The cause of this variability is not completely understood, but certain aspects of the parasite and of the host are involved (Andrade *et al*., 1999; Macedo *et al*., 2002).

Various studies have demonstrated that the strains of this parasite are composed of a variety of subpopulations with distinct characteristics between them and that natural populations of *T. cruzi* show great heterogeneity at the biological, biochemical, immunological and genetic levels (Deane *et al.,* 1984; Araújo e Chiari, 1988; Carneiro *et al.,* 1990; Brener, 1992; Oliveira *et al.,* 1997; Gomes *et al*., 1998a). Once confirmed this heterogeneity, Tibayrenc e Ayala (1988) verified that this species shows a predominantly clonal structure and evolution, with rare or no sexual activity, therefore keeping itself stable through time and space. Nevertheless, various authors have already found a correlation between zymodeme and biological properties for different strains of *T. cruzi*, including resistance/susceptibility to drugs (Andrade *et al.,* 1983; Andrade e Magalhães, 1997).

Although the possibility of working with cloned populations of *T. cruzi* is in fact a reality (Lauria-Pires *et al.,* 1997; Lana *et al.,* 1998; Revollo *et al.,* 1998; Toledo *et al.,* 2002), the truth is that the populations responsible for the infections in humans, triatomines and all the other species of mammals that function as vertebrate hosts of *T. cruzi*, are for the most part genetically heterogeneous (polyclonal) (Macedo *et al.,* 2002).

Advances in knowledge about the *T. cruzi* population genetics (Tibayrenc e Ayala, 1988) and molecular biology have clarified some reasons for the behaviour variability of natural populations of this species. Moreover, studies were carried out with strains demonstrating a correlation between the zymodemes and biology of the parasite (Andrade *et al.,* 1983; Andrade e Magalhães, 1997). Although most of studies with clone stocks of *T. cruzi*, pertaining to the four genotypes or principal ubiquitous zymodemes, have demonstrated a strong correlation between genetic distance and their fundamental biological characteristics (Laurent *et al.,* 1997; Lana *et al.,* 1998; Revollo *et al.,* 1998; Toledo *et al.,* 2002; 2003), Villareal *et al*. (2004) have observed a lack of correlation between susceptibility to benznidazole and phylogenetic diversity.

It was recently demonstrated that strains of *T. cruzi*, isolated in the state of Paraná from sylvatic reservoirs, triatomines and humans, grouped differently into two genetic groups, *T. cruzi* I and *T. cruzi* II (Zalloum *et al.,* 2005), conforming to the observations of other authors studying populations of the parasite originating from other geographical regions. The objective of this paper is to study the relation between the genetic and biological diversity of natural populations of *T. cruzi*, grouped as *T. cruzi* I and *T. cruzi* II (RSM, 1999) isolated from different hosts in northwest Parana state.

Material and methods

Strains of *T. cruzi*

Figure 1 shows the strains studied, the genetic groups they belong and the hosts they were isolated from. After defrosting, the strains were kept in Liver Infusion and Tryptose (LIT) medium, pH 7.2, with 10% of bovine fetal serum (Camargo, 1964), until a sufficient quantity of parasites were obtained to carry out the experiments. For the purpose of statistical comparison and tabulation, the results of each strain were grouped according to host H, G or T. These strains were characterised by RAPD and SSP-PCR with four genetic markers, grouping together strains referring to *T. cruzi* I - Sylvio and *T. cruzi* II - Esmeraldo (Zalloum *et al*., 2005). The four strains isolated from humans (H) were classified as *T. cruzi* II and the eight strains obtained from sylvatic reservoirs (G) and from triatomines (T) were classified as *T. cruzi* I.

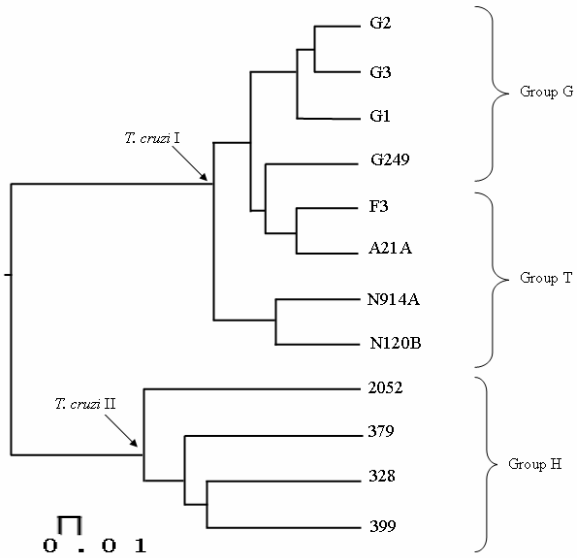

Figure 1: Phenogram of twelve strains of *Trypanosoma cruzi* constructed by the method of UPGMA (arithmetic mean, not weighted mean) using a matrix of genetic distance obtained by RAPD (randomly amplified polymorphic DNA) using the mean of three initiators (Zalloum *et al.,* 2005). They initiated the genetic groups which contain the strains (*T. cruzi* I and II) and the hosts from which they were isolated (H = humans in the chronic phase of Chagas Disease, G = sylvatic reservoirs and T = triatomines).

Culture Mediums

The mediums LIT and M16 (Chiari *et al.,* 1980) were used. LIT medium is a complex monophasic medium used for the maintenance of *T. cruzi*. M16 medium is poor in nutrients, pH 6.7, and favours the transformation of epimastigotes forms into metacyclic trypomastigotes.

Determination of the kinetic of growth and of metacyclogenesis

Parasites in an exponential phase of growth in LIT medium were inoculated in 15 mL of LIT medium and in 15 mL of M16 medium with a final concentration of 1.5×10^7 parasites/mL. The cultures were maintained at 28ºC. The growth of the parasites was measured 0, 12, 24, 36 and 48 hours after incubation. After this period, measurements were taken every two days until the 12th day of cultivation. The experiments were carried out in triplicate and the counts were done in a Neubauer chamber. Metacyclogenesis was measured in smears prepared with an aliquot of 50 µL of culture collected 0, 2, 4, 6, 8, 10 and 12 days after cultivation. The smears were stained by the technique of Panotic (Newprov-Curitiba, Estado do Paraná). Five hundred forms without selection were counted, using an optic microscope in amplifications of 1000× and percentages of transition forms of epimastigotes-trypomastigotes and trypomastigotes were calculated.

Infectivity of culture trypomastigotes forms

For each strain, 2×10^6 metacyclic trypomastigotes forms in the eighth day of cultivation in M16 medium were intraperitoneally inoculated into male BALB/c mice of four weeks of age. Infectivity was determined by polymerase chain reaction (PCR) according to the technique of Gomes *et al.* (1998 a) and expressed in percentage of positive mice.

Determination of susceptibility to benznidazole

Parasites in an exponential phase of growth in LIT medium were inoculated under the action of benznidazole (ROCHE, Lot no. BS01070001, 99.8% purity) in concentrations that varied from 0.25 to 500 µg/mL. The drug was dissolved in a solution of LIT with 0.05% of dimethylsulfoxide (DMSO) (Wittner *et al.,* 1982). Preliminary experiments showed that the DMSO in this concentration was not toxic for the strains of *T. cruzi* used in this study. The cultures were maintained at 28ºC. The experiments were carried out in triplicate. The counting of the parasites was carried out by the technique of Brener (1962) after 24 and 48 hours of incubation. The action of the benznidazole was evaluated by means of comparison with a control group without the drug (Ferreira *et al*., 2004), being determined by IC50-24 and IC50-48 (the concentration at which 50% of the parasites were killed after 24 and 48 hours of contact). The maximum percentages of growth and of inhibition were also determined according to Ferreira *et al.* (2004).

Parameters evaluated

Table 1 shows the parameters studied and their abbreviations.

Statistical analysis

The analysis of the results was carried out using the program SAS (Statistical Analysis System) – version 8.02 by the MANOVA/Duncan and Wilks'Lambda, Kruskal-Wallis and Kolmogorov-Smirnov tests, to the significance level of 5%.

Ethics Committee Evaluation

This study was approved by the Ethics Committee for Animal Experimentation (ECAE) of the State University of Maringa, State of Paraná.

Table 1. Relation of Parameters studied and abbreviations.

Parameters related to growth kinetic	Abbreviations
Number of parasites on the 6th day of growth in culture in LIT medium	NP6-LIT
Number of parasites on the 6th day of growth in culture in M16 medium	NP6-M16
Number of parasites on the 12th day of growth in culture in LIT medium	NP12-LIT
Number of parasites on the 12th day of growth in culture in M16 medium	NP12-M16
Doubling time of parasites in culture in LIT medium	DT-LIT
Doubling time of parasites in culture in M16 medium	DT-M16
Parameters related to the kinetic of metacyclogenesis	
Highest % of trypomastigotes in culture in LIT medium	>%Try-LIT
Highest % of trypomastigotes in culture in M16 medium	>%Try-M16
Maximum number of trypomastigotes in culture in LIT medium	MNTry-LIT
Maximum number of trypomastigotes in culture in M16 medium	MNTry-M16
Day with highest % of trypomastigotes in LIT medium	D>%Try-LIT
Day with highest % of trypomastigotes in M16 medium	D>%Try-M16
Highest % of transition forms in LIT medium	>%Tra-LIT
Highest % of transition forms in M16 medium	>%Tra-M16
Maximum number of transition forms in LIT medium	MNTra-LIT
Maximum number of transition forms in M16 medium	MNTra-M16
Day with highest % of transition forms in LIT medium	D>%Tra-LIT
Day with highest % of transition forms in M16 medium	D>%Tra-M16
Infectivity of culture trypomastigotes in mice	%Inf-PCR
Parameters related to susceptibility to benznidazole	
IC50 after 24 hours under the action of benznidazole	IC50-24
Maximum % of inhibition after 24 hours under the action of benznidazole	M%I-24
Maximum % of growth after 24 hours under the action of benznidazole	M%G-24
IC50 after 48 hours under the action of benznidazole	IC50-48
Maximum % of inhibition after 48 hours under the action of benznidazole	M%I-48
Maximum % of growth after 48 hours under the action of benznidazole	M%G-48

Results

The results for the parameters, in which strains of the group *T. cruzi* II (H) were different from strains of the group *T. cruzi* I (G and/or T), are shown below.

Growth kinetic

Table 2 shows the means and the standard deviations of the parameters related to the growth kinetic of the three groups studied. The comparison between the means of the groups for all the parameters showed that there is a significant difference ($p<0.0001$) between the groups of strains *T. cruzi* I (G and T) and *T. cruzi* II (H). The group *T. cruzi* II (H) presented a significant difference in three of the six parameters related to the growth kinetic. According to these three parameters, group *T. cruzi* II (H) was statistically different from group *T. cruzi* I (G or T) in the NP6-LIT, NP12-LIT and DT-LIT parameters. In the LIT medium, the final average growth was five times greater than the growth obtained in the M16 medium ($p=0.0006$ for H, $p<0.0001$ for G and $p=0.007$ for T). Group *T. cruzi* II (H) showed an average final growth in the LIT medium as lower as in the M16 (12th day of the experiment), although the difference was statistically significant only in relation to group T in LIT medium ($p=0.0397$). In LIT medium, group *T. cruzi* II (H) showed a lower growth than group *T. cruzi* I (G) ($p=0.0065$) on the sixth day, and lower than (T) on the 12th day of cultivation ($p=0.0397$). Group *T. cruzi* II (H) showed a significantly greater doubling time than that of group *T. cruzi* I (G) ($p=0.0079$) in LIT medium. In M16 medium, the doubling time for group *T. cruzi* II (H) was greater than that of *T. cruzi* I (G and T), although not significantly.

Table 2. Means and standard deviations of parameters derived from the growth kinetic, compared among the groups of strains *T. cruzi* II, isolated from chronic chagasic patients (H), and *T. cruzi* I, isolated from sylvatic reservoirs (G) and triatomines (T).

Parameters	Mean value and standard deviation/group			Probability (p)	Duncan Test
	H	G	T		
NP6-LIT ($\times10^6$ parasites/mL)	109.07 ± 20.16	135.25 ± 31.10	103.58 ± 18.74	0.0065*	(H=T)≠G
NP6-M16 ($\times10^6$ parasites/mL)	33.50 ± 9.19	33.08 ± 14.00	41.58 ± 23.39	0.3792	H=G=T
NP12-LIT ($\times10^6$ parasites/mL)	261.25 ± 46.09	266.67 ± 61.40	327.00 ± 86.98	0.0397*	(H=G)≠T
NP12-M16 ($\times10^6$ parasites/mL)	45.50 ± 23.09	54.08 ± 41.00	49.83 ± 23.27	0.7872	H=G=T
DT-LIT (hours)	43.24 ± 11.86	27.34 ± 14.10	37.27 ± 8.54	0.0079*	(H=T)≠G
DT-M16 (hours)	87.40 ± 51.56	76.35 ± 84.98	46.18 ± 43.28	0.2623	H=G=T

* Significant difference at $p<0.05$

Kinetic of metacyclogenesis

Table 3 shows the percentile results and Table 4 and Figure 2 show the numeric results obtained for the kinetic of growth and metacyclogenesis in LIT and M16 mediums for the three groups studied. Group *T. cruzi* II (H) showed a significant difference in nine of the 13 parameters related to metacyclogenesis (Tables 3 and 4).

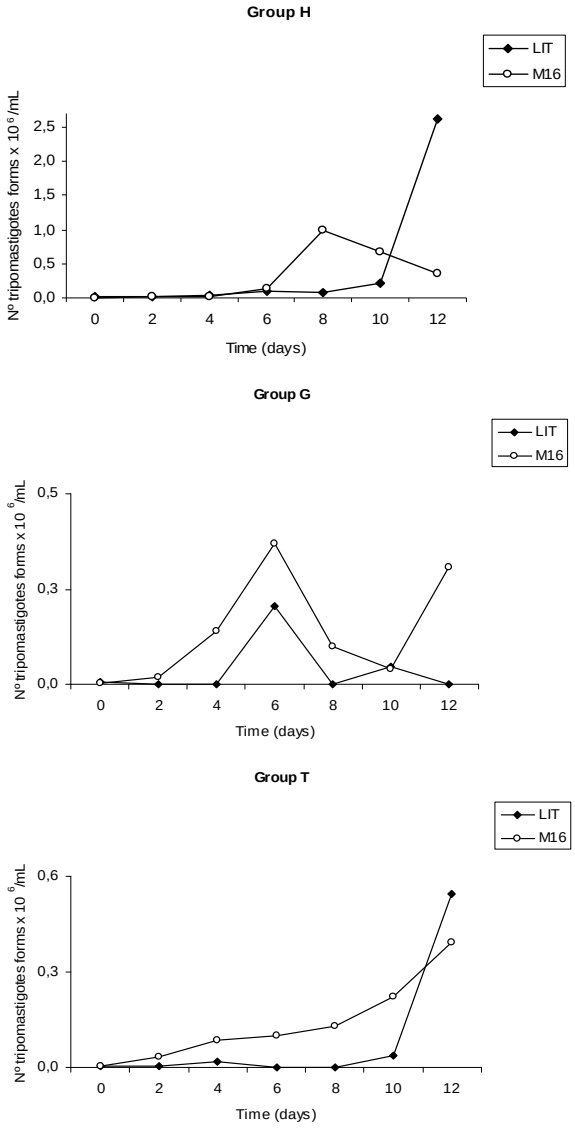

Figure 2. Mean number of trypomastigotes forms in LIT and M16 medium for the groups of strains of *T. cruzi* II, isolated from humans (Group H) and *T. cruzi* I, isolated from sylvatic reservoirs – *Didelphis sp* (Group G) and triatomines – *Triatoma sordida* and *Panstrongylus megistus* (Group T).

Table 3. Means and standard deviations of the parameters derived from the kinetic of metacyclogenesis, compared among the groups of strains of *T. cruzi* II, isolated from chronic chagasic patients (H), and *T. cruzi* I, isolated from sylvatic reservoirs (G) and triatomines (T).

Parameters	Mean value and standard deviation/group			Probability (p)	Duncan Test
	H	G	T		
>%Try-LIT	0.73±0.78	0.13±0.30	0.22±0.26	0.0120*	H≠(G=T)
>%Try-M16	2.45±3.53	0.97±0.80	0.77±0.57	0.1228	H=G=T
D>%Try-LIT	9.00±5.43	1.50±2.71	8.01±5.88	0.0011*	(H=T)≠G
D>%Try-M16	9.00±1.04	5.00±1.00	12.00±0.00	<0.0001*	H≠G≠T
>%Tra-LIT	51.13±7.08	48.01±8.10	30.81±18.00	0.0005*	(H=G)≠T
>%Tra-M16	47.25±28.39	42.56±11.00	27.98±9.44	0.0393*	H=G; G=T; H≠T
D>%Tra-LIT	5.00±1.81	4.00±0.00	3.15±1.32	0.0056*	H=G; G=T; H≠T
D>%Tra-M16	6.00 ± 1.48	8.50 ± 3.70	4.16 ± 1.99	0.0011*	(H=T) ≠G
%Inf-PCR	33.35 ± 20.41	50.00 ± 5.99	37.50 ± 27.29	0.4770	H=G=T

* Significant difference at $p<0.05$

Table 4. Means and standard deviations of the parameters derived from the kinetic of growth and of metacyclogenesis, compared among the groups of strains of *T. cruzi* II, isolated from chronic chagasic patients (H), and *T. cruzi* I, isolated from sylvatic reservoirs (G) and triatomines (T).

Parameters	Mean value and standard deviation/group			Probability (p)	Duncan Test
	H	G	T		
MNTry-LIT ($\times 10^6$/mL)	1.98 ± 2.32	0.19 ± 0.40	0.53 ± 0.34	0.0071⋆	H≠(G=T)
MNTry-M16 ($\times 10^6$/mL)	1.14 ± 2.08	0.40 ± 0.60	0.45 ± 0.29	0.2838	H=G=T
MNTra-LIT ($\times 10^6$/mL)	43.93 ± 24.32	35.50 ± 8.90	18.57 ± 14.45	0.0033⋆	(H=G)≠T
MNTra-M16 ($\times 10^6$/mL)	15.12 ± 13.10	16.51 ± 6.30	11.03 ± 4.86	0.3020	H=G=T

⋆ Significant difference at $p<0.05$

In these nine parameters, group *T. cruzi* II (H) was statistically different from group *T. cruzi* I (G and T) in three parameters and statistically different from group *T. cruzi* I (G or T) in six parameters. The production of trypomastigotes occurred more rapidly in M16 medium than in LIT for both groups *T. cruzi* I and *T. cruzi* II (Figure 2). In LIT medium, the *T. cruzi* II (H) produced a greater percentage ($p=0.0120$) of trypomastigotes than the *T. cruzi* I (G and T). This percentage occurred in the ninth day for group *T. cruzi* II (H) ($p=0.0011$) and between the first and the second day for group *T. cruzi* I (G) (Table 3). In M16 medium, group *T. cruzi* II (H) produced a greater percentage of trypomastigotes than group *T. cruzi* I (G and T), although the difference was not significant (Table 3). This greater percentage of trypomastigotes in group H occurred at an intermediate length of time, different from groups G and T (H≠G≠T) ($p<0.0001$). During the evaluation of the metacyclogenesis, forms in the epimastigotes-trypomastigotes transition phase were also observed. Group *T. cruzi* II (H) presented a greater percentage of forms in epimastigotes-trypomastigotes transition than group *T. cruzi* I (T), in both LIT medium ($p=0.0005$) and M16 medium ($p=0.0393$) (Table 3).

Infectivity of culture trypomastigotes forms

The infectivity of culture forms evaluated by the PCR technique did not present a significant difference between the groups *T. cruzi* I and *T. cruzi* II (H=G=T) (Table 3).

Susceptibility to benznidazole

Table 5 shows the means and standard deviations of the parameters related to the susceptibility to benznidazole for the three groups studied. Group *T. cruzi* II (H) was significantly different from group *T. cruzi* I (T) in one of the six parameters related to the susceptibility to benznidazole. Susceptibility to benznidazole, after 24 hours of parasite-drug contact, did not produce a significant difference between the groups *T. cruzi* I and *T. cruzi* II (H=G=T) for the three parameters evaluated. However, after 48 hours of contact, the group *T. cruzi* II (H) showed a lower resistance (greater susceptibility) to benznidazole, expressed by a lower IC50-48, when compared with group *T. cruzi* I (T) ($p=0.0033$). There was no significant difference for the parameters M%I-48 and M%C-48.

Table 5. Means and standard deviations of the parameters derived from the susceptibility to benznidazole *in vitro*, compared among the groups of strains of *T. cruzi* II, isolated from chronic chagasic patients (H), and *T. cruzi* I, isolated from sylvatic reservoirs (G) and triatomines (T).

Parameters	Mean value and standard deviation/group			Probability (p)	Duncan Test
	H	G	T		
IC50-24 (µg/mL)	115.68 ± 118.78	86.80 ± 83.80	153.44 ± 163.67	0.4412	H=G=T
M%I-24	75.05 ± 18.64	75.51 ± 17.80	82.10 ± 17.86	0.5711	H=G=T
M%G-24	19.67 ± 15.04	17.82 ± 26.00	14.32 ± 29.88	0.8628	H=G=T
IC50-48 (µg/mL)	5.27 ± 5.59	1.80 ± 1.50	18.28 ± 19.05	0.0033⋆	(H=G)≠T
M%I-48	99.85 ± 0.33	100.00 ± 0.00	92.09 ± 27.33	0.3839	H=G=T
M%G-48	9.35 ± 12.82	20.39 ± 22.50	10.48 ± 28.59	0.4194	H=G=T

⋆ Significant difference at $p<0.05$

Discussion

Over the years, various studies of *T. cruzi* and Chagas' disease have searched for an explanation to the diversity of the possible clinical forms during the evolution of the infection. One of the ways was to correlate the biology of the parasite with clinical disease forms. Studies developed the way of correlating the biology of the protozoan with its biochemical and genetic parameters (Andrade, 1974; Andrade *et al.,* 1983; Andrade e Magalhães, 1997; Carneiro *et al.,* 1991; Steindel *et al.,* 1995). The improvement of materials and methods such as isogenic animals, cloning of parasites, the employment of molecular techniques and statistical methods in studies of the genetics of the *T. cruzi* population, some studies (Barnabé *et al.,* 1983; Lana *et al.,* 1998; Revollo *et al*., 1998; Toledo *et al.,* 2002) have managed to demonstrate the existence of a correlation between the biology and genetics of the protozoan, including the parameter of susceptibility/resistance to drugs, (Andrade e Magalhães, 1997; Murta *et al.,* 1988), and also verify that the clones of the group *T. cruzi* I are more resistant to benznidazole than the clones of group *T. cruzi* II (Revollo *et al*., 1998; Toledo *et al.,* 2003; 2004). However, Villareal *et al*. (2004), even though working with cloned population, have not demonstrated significant correlation between *in vitro* susceptibility to benznidazole and phylogenetic diversity.

The present paper aimed to study the relation between the genetic and biological diversity of natural populations of *T. cruzi*, isolated from different hosts from the state of Parana, Brazil, grouped as *T. cruzi* I

and *T. cruzi* II.

The data obtained in this study showed that there are discreet differences in the biological behaviour of natural populations of *T. cruzi* pertaining to the large genetic groups *T. cruzi* I and *T. cruzi* II (Zalloum *et al*., 2005), isolated from different hosts (humans, sylvatic reservoirs and vector triatomines) when the kinetic of growth and of metacyclogenesis, and susceptibility to benznidazole were compared.

When considering the three host groups (H, G and T), the comparison between the means of each parameter showed that group *T. cruzi* II (H) is significantly different from group *T. cruzi* I (G and T) in 13 of the 25 parameters studied. Group *T. cruzi* II (H) differed from group *T. cruzi* I (both G and T) in 3 parameters, and from *T. cruzi* I (G or T) in ten parameters.

Among the parameters studied in the growth kinetic, group *T. cruzi* II (H) was statistically different from group *T. cruzi* I (G or T) in 3 of the 6 parameters analysed; i.e. in 50% of them. This result shows the importance of the type of experiment and culture medium used for the comparison of *T. cruzi* from different sources. In LIT medium, the average final growth was five times greater than the growth obtained in M16 medium. This result was expected, since M16 medium is an impoverished medium that induces nutritional stress, promoting metacyclogenesis (Chiari *et al.,* 1980). In LIT medium, to the same extent as in M16 medium, group *T. cruzi* II (H) showed lower growth in both evaluations for growth kinetic, in relation to group *T. cruzi* I (G or T). Similarly, group *T. cruzi* II (H) showed a doubling time in LIT medium as great as in M16 medium, which reflects slower growth. These findings, here obtained by the study of strains, are in agreement with the observations by Laurent *et al.* (1997); Lana *et al.* (1998) and Revollo *et al.* (1998), which demonstrate that clones pertaining to the group *T. cruzi* II show lower multiplication in *T. infestans,* LIT medium and Vero cells, respectively. A biological implication of these findings is that the slow growth may make diagnosis difficult in parasitological tests, leading to false negative results. Furthermore, experimental studies carried out *in vivo*, using clones and isogenic animals, showed that parasites from the group *T. cruzi* II presented a lower infection rate in organs and tissues, as well as produced lower numbers of inflammatory reactions, than clones of the group *T. cruzi* I (Toledo *et al.*, 2002), which confirms that groups with lower growth may be less pathogenic.

The fact that group G (*T. cruzi* I) showed greater growth in the 6th day of cultivation in LIT medium and group T (*T. cruzi* I) showed it in the 12th day may be related to exhaustion of the medium and production of metabolites, which certainly influenced the final results of the kinetic of growth. However, the fact that *T. cruzi* I grows more rapidly and in greater number than *T. cruzi* II is preserved. This data is confirmed by results observed in the calculation of doubling time, where the greater capacity of multiplication in less time of group *T. cruzi* I (G) is clear.

Among the parameters referring to metacyclogenesis, group *T. cruzi* II (H) was statistically different from group *T. cruzi* I (G and/or T) in 9 of the 13 parameters analysed, i.e. in 69.2% of them, showing the importance of these parameters in comparing *T. cruzi* from different sources. Group *T. cruzi* II (H) produced a greater number of trypomastigotes and transition forms more rapidly in M16 medium than in LIT medium. This result is in agreement with others from previous literature considering that 1) the production of epimastigotes-trypomastigotes transition forms and consequently of metacyclic trypomastigotes is an intrinsic characteristic of the strain (Chiari, 1981; Araújo *et al.,* 1999) and 2) the medium that facilitates metacyclogenesis will do it earlier with the group that produces more trypomastigotes (*T. cruzi* II - H). Group *T. cruzi* II (H) produced a greater percentage of trypomastigotes in LIT medium than group *T. cruzi* I (G and T), and a greater percentage of epimastigotes-trypomastigotes transition forms in both LIT and M16 medium than group *T. cruzi* I (T). Although the *in vitro* experiments in acellular medium simulate the stage of the cycle of *T. cruzi* that happens in the intestinal tube of the triatomines, these data do not support the reports by Lana *et al.* (1998), who observed clones pertaining to the genetic group *T. cruzi* I producing more metacyclogenesis *in vivo* (intestinal tube of *T. infestans).* The difference in behaviour encountered may be explained by the fact that the two results were produced in distinct conditions or maybe by the fact that Lana *et al.* (1998) worked with cloned populations.

Curiously, during the evaluation of metacyclogenesis, high percentages similar to those of the epimastigotes-trypomastigotes transition forms were observed for all the groups, in both LIT and M16 mediums, that did not convert themselves into trypomastigotes forms, even when the experiment was continued. Martínez-Díaz *et al*. (2001) also reported high percentages of epimastigotes-trypomastigotes transition forms, but they converted themselves into trypomastigotes when observed for longer time. Araújo *et al.* (1999), evaluating some of the strains used in this study, reported that these strains had a low capacity to form trypomastigotes, although they reported percentages slightly higher than those described here. Ávila *et al.* (2003) assert that biochemical changes in the membrane of the parasite, as well as changes in their

gene expression, precede morphological transformations of *T. cruzi* during the process of differentiation. Based on these data some hypotheses were raised to explain the high production of epimastigotes-trypomastigotes transition forms that do not convert themselves into trypomastigotes. Because these strains are of low metacyclogenesis capacity, it may be assumed that the process did not complete itself due to the quality of the medium used and/or the time of cultivation in the laboratory, or even, their intrinsic characteristics.

Together, the findings relating to the greatest production of metacyclic trypomastigotes and epimastigote-trypomastigote transition forms observed for group *T. cruzi* II (H) might explain the lower growth capacity of this group, reducing the possibility of positivation of the parasitological methods (hemoculture), as the trypomastigote and epimastigote-trypomastigote transition forms do not reproduce themselves.

In this study, a statistical difference in infectivity between the groups *T. cruzi* I and II was not observed. This finding differs from that of Toledo *et al*. (2002), who demonstrated a lower percentage of infectivity for the clones of group *T. cruzi* II. This difference might be explained by the use in this study of technology that is clearly more sensitive (Bértoli *et al.,* 2005; Miyamoto *et al.,* 2005) than that which was used by those authors to measure infectivity.

Among the six parameters evaluated for susceptibility to benznidazole, group *T. cruzi* II (H) was statistically different from group *T. cruzi* I (T) in only 1 parameter (16%), showing that in the conditions developed in this study, the parameters derived from the study of susceptibility to benznidazole were not among the most important to differentiate *T. cruzi* of different sources. This may be due to the great heterogeneity of behaviour observed in each of these large groups of *T. cruzi*. Even in studies carried out with cloned stocks and smaller subdivisions of *T. cruzi*, various authors found large standard deviations in many of the parameters analysed (Laurent *et al.*, 1997; Lana *et al.*, 1998; Revollo *et al*., 1998; Toledo *et al.*, 2002, 2003, 2004). Villarreal *et al*. (2004) observed lack of significant correlation between susceptibility to benznidazole and phylogenetic diversity. Group *T. cruzi* II (H) showed greater susceptibility to benznidazole than group *T. cruzi* I (T), expressed by a lower IC50-48, suggesting that strains which reproduce themselves more rapidly (*T. cruzi* I) might impair the action of benznidazole. These data are in agreement with Barnabé *et al.* (1983) and Revollo *et al*. (1998) who observed *in vitro* a greater susceptibility to benznidazole and nifurtimox for the clones of the group *T. cruzi* II when they studied epimastigotes and amastigotes. These results are also in agreement with experiments *in vivo* carried out with clones of *T. cruzi* in isogenic mice (Toledo *et al*., 2003, 2004).

Results of the present study allow us to suggest a weak relation between the genetic and biological diversity of natural populations of *T. cruzi* isolated from different hosts.

References

ANDRADE, L.O. *et al.* Differential tissue distribution of diverse clones of *Trypanosoma cruzi* in infected mice. *Mol. Biochem. Parasitol.,* Amsterdam v. 100, n. 2, p. 163-172, 1999.

ANDRADE, S.G. Caracterização de cepas do *Trypanosoma cruzi* isoladas no Recôncavo Baiano. *Rev. Pat. Trop.,* Goiânia, v. 1, p. 65-121, 1974.

ANDRADE, S.G.; MAGALHÃES, J.B. Biodemes and zymodemes of *Trypanosoma cruzi* strains: correlation with clinical data and experimental pathology. *Rev. Soc. Bras. Med. Trop.,* Uberaba, v. 30, p. 27-35, 1997.

ANDRADE, V. *et al*. Correlation between isoenzyme patterns and biological behavior of different strains of *Trypanosoma cruzi*. *Trans. R. Soc. Trop. Med. Hyg.,* London, v. 77, p. 796-799, 1983.

ARAÚJO, S.M.; CHIARI, E. Caracterização biológica de clones das cepas Y, CL e MR de *Trypanosoma cruzi* em camundongos C3H isogênico. *Mem. Inst. Oswaldo Cruz,* Rio de Janeiro, v. 83, p. 175-181, 1988.

ARAÚJO, S.M. *et al.* Biology of *Trypanosma cruzi* strains isolated from chagasic patients from different geographic origins residing in northwestern region of the state of Parana, Brasil. *Acta Sci.,* Maringá, v. 21, n. 2, p. 229-235, 1999.

ÁVILA, A.R.V. *et al*. Stage-sepecific gene expression during *Trypanosoma cruzi* metacyclogenesis. *Genet. Mol. Res*., Ribeirão Preto, v. 2, n. 1, p. 159-168, 2003.

BARNABÉ, C. *et al*. *Trypanosoma cruzi*. A pharmacological comparison of some bolivian isoenzymic strains. *Ann. Soc. Belg. Med. Trop.,* Antuérpia, v. 63, p. 319-324, 1983.

BÉRTOLI, M. *et al*. Infectividade de cepas de *Trypanosoma cruzi* I e II isoladas de diferentes hospedeiros. *Rev. Soc. Bras. Med. Trop.,* Uberaba, v. 38, n. 1, p. 312-313, 2005.

BRENER, Z. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi. Rev. Inst. Med. Trop. São Paulo,* São Paulo v. 4, p. 389-396, 1962.

BRENER, Z. *Trypanosoma cruzi*: taxonomy, morphology and life cycle. *In:* WENDEL S. *et al.* (Ed.). Chagas disease (American Trypanosomiasis): Its impact on transfusion and Clinical Medicine. ISBT Brazil, São Paulo, *Soc. Bras. Hem. Hemoter.,* Rio de Janeiro, p. 13-29, 1992.

CAMARGO, E.P. Growth and differentiation in *Trypanosoma cruzi* I. Origin of metaciclic tripanosomes in liquid media. *Rev. Inst. Med. Trop.* São Paulo, São Paulo, v. 6, p. 93-100, 1964.

CAMARGO, M.E. *et al*., Inquérito sorológico da prevalência da infecção chagásica no Brasil, 1975-1980. *Rev. Inst. Med. Trop. São Paulo,* São Paulo, v. 26, p. 192-204, 1984.

CARNEIRO, M. *et al.* Changes in the isoenzyme and kinetoplast DNA patterns *of Trypanosoma cruzi* strains induced by maintenance in mice. *Acta Trop.,* Amsterdam, v. 47, p. 35-45, 1990.

CARNEIRO, M. *et al*. Biological characterization of *Trypanosoma cruzi* strains from different zymodemaes and schizodemes. *Mem. Inst. Oswaldo Cruz,* Rio de Janeiro, v. 86, p. 387-393, 1991.

CHIARI, E. *Diferenciação do Trypanosoma cruzi em cultura.* 1981. Dissertação (Doutorado)-Universidade Federal de Minas Gerais, Belo Horizonte, 1981.

CHIARI, E. *et al*. Meios empobrecidos em nutrientes na diferenciação epimastigota-tripomastigota do *Trypanosoma cruzi*. *In*: REUNIÃO ANUAL EM PESQUISA BÁSICA EM DOENÇA DE CHAGAS, 7., 1980. Caxambu. *Anais...* Caxambu, 1980. p. 41.

DEANE, M.P. *et al. Trypanosoma cruzi*: inoculation schedules and re-isolation methods select individual strains from doubly infected mice, as demonstrated by schizodeme and zimodeme analyses. *J. Parasitol.,* Seoul, v. 31, n. 2, p. 276-280, 1984.

DIAS, J.C.P. Epidemiology of Chagas disease. *In:* WENDEL S. *et al.* (Ed.). Chagas disease (American Trypanosomiasis): Its Impact on Transfusion and Clinical Medicine. ISBT BRAZIL-SBHH, São Paulo, 1992. p. 49-80.

DIAS, J.C.P. Present situation and future of human Chagas' disease in Brazil. *Mem. Inst. Oswaldo Cruz,* Rio de Janeiro, v. 92, supl. I, p. 13-15, 1997.

FERREIRA, I.C.P. *et al*. Anti-leishmanial activity of alkaloidal extract from *Aspidosperma ramiflorum*. *Mem. Inst. Oswaldo Cruz,* Rio de Janeiro, v. 99, n. 3, p. 325-327, 2004.

GOMES, M.L. *et al*. Genetic relationships between *Trypanosoma cruzi* strains isolated from chronic patients in southern Brazil as revealed by RAPD and SSR-PCR analysis. *Acta Trop.,* Amsterdam, v. 69, p. 99-109, 1998a.

GOMES, M.L. *et al*. *Trypanosoma cruzi*: optimization of Polymerase Chain Reaction for detection in human blood. *Exp. Parasitol.*, Nova York, v. 88, p. 28-33, 1998b.

LANA, M. *et al. Trypanosoma cruzi*: campared vectorial transmissibility of three major clonal genotypes by *Triatoma infestans. Exp. Parasitol*., Nova York v. 90, p. 20-25, 1998.

LAURENT, J.P. *et al*. Impact of clonal evolution on the biological diversity of *Trypanosoma cruzi*. *Parasitol.,* Londres, v. 114, p. 213-218, 1997.

LAURIA-PIRES, L. *et al.* Diversity of *Trypanosoma cruzi* stocks and clones derived from Chagas disease patients: I-behavioral characterization *in vitro*. *Rev. Soc. Bras. Med. Trop.,* Uberaba, v. 30, n. 3, p. 187-192, 1997.

MACEDO, A.M. *et al.* Chagas disease: role of parasite genetic variation in pathogenesis. *Expert reviews en molecular medicine*. 5 march 2002. Disponível em*: <http://www-ermm.cbcu. cam.ac.uk*>. acesso em: 05 mar. 2002.

MARTÍNEZ-DÍAZ, R.A. *et al*. Biological Characterization of *Trypanosoma cruzi* Strains. *Mem. Inst. Oswaldo Cruz,* Rio de Janeiro, v. 96, n. 1, p. 53-59, 2001.

MIYAMOTO, C.T *et al.* Reação em Cadeia da Polimerase (PCR) na detecção de *Trypanosoma cruzi* em sangue da camundongos infectados com diferentes genótipos e tratados com benznidazol. *Rev. Soc. Bras. Med. Trop.,* Uberaba, v. 38, n. 1, p. 370, 2005.

MURTA, S.M.F. *et al*. Molecular characterization of susceptible and naturally resistant strain of *Trypanosoma cruzi* to Benznidazole and Nifurtimox. *Mol. Biochem. Parasitol.,* Amsterdam, v. 93, p. 203-214, 1988.

OLIVEIRA, R.P. *et al*. An alternative approach to evaluating the intraespecific genetic variability of parasites. *Parasitol. Today*, Oxford, v. 13, n. 5, p. 196-200, 1997.

REVOLLO, S. *et al. Trypanosoma cruzi*: impact of clonal evolution of the parasite on its biological and medical properties. *Exp. Parasitol.,* New York, v. 89, p. 30-39, 1998.

RSM - Recommendations from Satellite Meeting. *Mem. Inst. Oswaldo Cruz*, Rio de Janeiro, v. 94, supl. I, p. 429-432, 1999.

STEINDEL, M. *et al.* Biological and isoenzimatic characterization of *Trypanosoma cruzi* strains isolated from silvatic reservoirs and vectors from the state of Santa Catarina, Southern Brazil. *Acta Trop.,* Amsterdam, v. 60, n. 3, p. 167-177, 1995.

TIBAYRENC, M.; AYALA, F.J. Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas'disease: genetical, taxonomical and epidemiological significance. *Evolution*, Temple, v. 42, p. 277-292, 1988.

TOLEDO, M.J.O. *et al.* Impact of *Trypanosoma cruzi* clonal evolution on its biological properties in mice. *Exp. Parasitol,* New York, v. 100, p. 161-172, 2002.

TOLEDO, M.J.O. *et al.* Chemotherapy with benznidazole and itraconazole for mice infected with different *Trypanosoma cruzi* clonal genotypes. *Antimicrob. Agents Chemother.*, Washington, D.C., v. 47, p. 223-230, 2003.

TOLEDO, M.J.O. *et al*. Genetic diversity and drug resistance in *Trypanosoma cruzi*, the agent of Chagas disease. *Rec. Adv. In Antimicrob. Agents Chemother.,* Washington, D.C., v. 4, p. 11-22, 2004.

VILLARREAL, D. *et al.* Lack of correlation between *in vitro* susceptibility to benznidazole and phylogenetic diversity of *Trypanosoma cruzi*, the agent of Chagas Disease. *Exp. Parasitol*., New York, v. 108, n. 1-2, p. 24-31, 2004.

WHO-World Health Organization. Control of Chagas Disease, Technical Report Series No. 905, p. 109, 2002.

WITTNER, M. *et al. Trypanosoma cruzi*: colony formation and clonal growth in agar. *Exp. Parasitol.,* New York, v. 53, p. 255-261, 1982.

ZALLOUM, L. *et al.* Trypanosoma cruzi: Two genetic groups in Parana state, Southern Brazil. *Exp. Parasitol.,* New York, v. 111, n. 1, p. 55-8, 2005.

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