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Biology of *Trypanosoma cruzi* strains isolated from chagasic patients from different geographic origins residing in northwestern region of the state of Paraná, Brazil

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ABSTRACT. Thirty-one *Trypanosoma cruzi* strains isolated from chagasic patients from different geographic origins who were residing in the northwestern region of the state of Paraná at the time of the study. The strains were analyzed using biological parameters including infectivity of culture forms, parasitemia curves, mortality and histopathologic patterns in male C3H/He mice. In addition, metacyclogenic capacity and growth in LIT and M16 cultures were evaluated. From thirty strains infected mice, 27 had patent parasitemia and 3 had subpatent parasitemia. Of these 30 strains, 16 were maintained by blood passages (BP), 7 by alternate and post blood passages (AP/BP) and 7 by alternate passages (AP). Eight strains had parasitemia peaks of 10^3 parasites/ml and 15 strains had peaks of 10^4 - 10^6 trypomastigotes/ml. It was not possible to measure this parameter for 7 strains. Three strains (PR 182, PR 1055, PR 501) caused mortality in mice. Histopathologic analysis revealed amastigote nests in the heart and skeletal muscle for PR 2259 and PR 182 strains respectively. The eight strains analyzed for this parameter induced inflammatory reactions in various organs. Epimastigote→trypomastigote differentiation rates for 21 analyzed strains were for the most part low. All strains were considered to have low-to-medium virulence. This homogeneity of biological behavior could be related to the fact that only strains isolated from humans were analyzed. Shortly, we intend to analyze other parameters and study *T. cruzi* strains isolated from triatomines and reservoirs from the northwestern region of the state of Paraná to compare to the human strains studied here.

Key words: *Trypanosoma cruzi,* infectivity, mortality, parasitemia.

RESUMO. **Biologia de cepas de T*rypanosoma cruzi* isoladas de pacientes chagásicos de diferentes origens geográficas residentes no noroeste do Paraná.** Trinta e uma cepas de *Trypanosoma cruzi* isoladas de pacientes chagásicos de diferentes origens geográficas e residentes no noroeste do Paraná foram analisadas pelos parâmetros biológicos: infectividade das formas de cultura, perfil das curvas de parasitemia, mortalidade e padrão histopatológico em camundongos C3H/He machos. Foi ainda avaliada a capacidade de metaciclogênese e crescimento das formas de cultura nos meios LIT e M16. Trinta cepas foram infectantes para o camundongo, 27 com parasitemia patente e 3 com parasitemia subpatente. Das trinta cepas, 16 foram mantidas por passagens sanguíneas (PS), 7 por passagens alternadas e posterior passagens sanguíneas (PA/PS) e 7 por passagens alternadas (PA). Pico de parasitemia da ordem de 10^3 parasitas/ml foi observado para 8 cepas e da ordem de 10^4 e 10^6 tripomastigotas/ml para 15 cepas. Para 7 cepas não foi possível avaliar este parâmetro. Mortalidade foi observada para três cepas (PR 182, PR 1055, PR 501). A análise do padrão histopatológico mostrou ninhos de amastigotas no coração para a cepa PR 2259 e no músculo esquelético para a PR 182. Para as oito cepas estudadas foram observados infiltrados inflamatórios nos diferentes órgãos estudados. As taxas de diferenciação epimastigota→tripomastigota para as 21 cepas analisadas foram em sua maioria baixas. As cepas aqui estudadas foram consideradas de baixa e média virulência. Esta homogeneidade do comportamento biológico pode estar relacionada ao fato de que somente cepas isoladas de humanos foram estudadas. Na seqüência pretende-se analisar outros parâmetros e estudar amostras de *T. cruzi* isoladas de triatomíneos e reservatórios da região noroeste do Paraná comparando-as com as cepas de humanos aqui estudadas.

Palavras-chave: *Trypanosoma cruzi,* infectividade, mortalidade, parasitemia.

Trypanosoma cruzi, the causative agent of *Chagas* disease, circulates in nature between man, vectors and domestic and sylvatic reservoirs. *T. cruzi* strains isolated from different regions or different hosts can exhibit intraspecies variation in virulence, pathogenicity, blood-form morphology, parasitemia curves, host cell infectivity and drug treatment response (Brener 1985, 1992). Adding to the complexity of this parasite is the fact that environmental conditions can influence its biological and biochemical behavior which emphasizes the importance of studies with samples isolated from humans, sylvatic reservoirs or triatomines from different endemic regions (Andrade, 1974; Schlemper Jr., 1982; Carneiro *et al.,* 1991; Steindel, 1993; Fernandes, 1994; Coura *et al.,* 1995; Gonzalez *et al.,* 1995; Castro, 1997). Although Camargo's *et al.* (1984) national investigation estimated the prevalence of chagasic infection at 4% in the State of Paraná, data related to *Chagas* disease in this state is limited to surveys of human cases, infected triatomines and affected domiciles (Simões, 1943; Almeida, 1948; Lobo *et al.,* 1954; Souza-Araújo, 1954). In a serological survey of 2,700 individuals from five municipalities in the northwestern region of the state of Paraná, Gomes *et al.* (1992) found that 6.3% of individuals had positive *T. cruzi* serology and Toledo *et al.* (1997) evaluated the efficacy of benzonidazole in strains isolated from chagasic patients from the same region. In the present study we analyze biological characteristics of these strains including infectivity, parasitemia curves, mortality and histopathologic patterns in mice and growth and metacyclogenesis in axenic culture.

Material and methods

T. cruzi samples - Thirty-one samples were isolated by hemoculture in LIT medium (Chiari *et al.,* 1989) from chronic chagasic patients from different endemic regions (Pernambuco, Alagoas, Bahia, Minas Gerais, São Paulo and Paraná state) who were living in the northwestern region of the state of Paraná, Brazil at the time of the study (Table 1). Following isolation, the samples were maintained in LIT medium (Camargo 1964) for bi-weekly passages for a maximum of four months and then frozen in liquid nitrogen. During this period, aliquots were obtained for mouse inoculation and metacyclogenesis and growth studies.

Table 1. Biological behavior in mice of *Trypanosoma cruzi* samples isolated from chagasic patients from different geographic origins residing in northwest Paraná

T. cruzi sample	Region of origin	Parasitemia after first inoculation	Maintenance	Nº of AP necessary for adaptation in mice	Parasitemia peak (parasites/ml)	Mortality [a] (%)
PR 036	São Pedro Turvo/SP	PP	BP	0	10^5	0
PR 182	Itamarandiba/MG	PP	BP	0	10^5	12.5
PR 209	M. Claros/MG-Pres.Prudente/SP	PP	BP	0	10^5	0
PR 316	Minas Novas/MG	PP	BP	0	10^4	0
PR 373	Montes Claros/MG	PP	BP	0	10^5	0
PR 396	Lassance/MG	PP	BP	0	10^3	0
PR 401	Berile/MG	PP	BP	0	10^3	0
PR 402	Poté/MG	PP	BP	0	10^3	0
PR 427	Terra Branca/MG	PP	BP	0	10^3	0
PR 458	Florinea/SP	PP	BP	0	10^5	0
PR 501	Santa Cruz do Rio Pardo/SP	PP	BP	0	10^6	20
PR 655	Brasília de Minas/MG	PP	BP	0	10^4	0
PR 1055	Conceição do Piancó/PB	PP	BP	0	10^4	25
PR 1256	União dos Palmares/AL	PP	BP	0	10^5	0
PR 1921	Iepê/SP	PP	BP	0	10^4	0
PR 2259	Virgem da Lapa/MG	PP	BP	0	10^6	0
PR 076	Coração de Jesus/MG	PP	AP/BP	4	10^4	0
PR 149	Montes Claros/MG	PP	AP/BP	4	10^4	0
PR 184	Montes Claros/MG	PP	AP/BP	1	10^3	0
PR 192	Sto. Antônio da Alegria/SP	PP	AP/BP	5	10^4	0
PR 379	Londrina/PR	PP	AP/BP	5	10^3	0
PR 399	1° de Maio/PR	PP	AP/BP	5	10^3	0
PR 443	?	PP	AP/BP	1	10^3	0
PR 101	Capelinha da Graça/MG	SP	AP	-	-	0
PR 114	S. João do Paraíso/MG	PP	AP	-	-	0
PR 150	Januária/MG	SP	AP	-	-	0
PR 328	Congoninhas/PR	PP	AP	-	-	0
PR 367	Teófilo Otoni/MG	PP	AP	-	-	0
PR 387	Sebastião Laranjeira/BA	PP	AP	-	-	0
PR 2052	Mirasselva/PR	SP	AP	-	-	0
PR 168	Catuni/MG	NI	-	-	-	-

a: up to 120 days of infection. BP: blood passages; AP: alternate passages (mouse-hemoculture-mouse); PP: patent parasitemia; SP: subpatent parasitemia; NI: not infected

Animals - Four-week-old male C3H/He mice from the Clinical Analysis Department of the State University of Maringá were used.

Infection and maintenance of samples in mice - Metacyclic trypomastigotes (1×10^7) collected on the 6th day of growth in M16 medium (Chiari *et al.,* 1980) were inoculated i.p. for the first infection of each sample. After this inoculation, the samples were divided into two groups. Samples that determined patent parasitemia were maintained by

serial blood passages with intervals and inocula determined according to each strain's individual characteristics. Samples that determined subpatent parasitemia or very low patent parasitemia were maintained by alternate passages (mouse-hemoculture-mouse) in order to attain a sufficient number of parasites for serial blood passages. In alternate passages, the inoculated mice were examined every two days for 30 days, at which time a hemoculture was performed. After 20 days the positive hemocultures were again inoculated in mice, constituting one cycle. For samples that required this procedure, a maximum of five cycles were performed.

Strain characterization

Culture form infectivity - Fresh blood examination was used to confirm patent parasitemia and hemoculture was used to confirm subpatent parasitemia.

Parasitemia curves - After an adaptation period consisting of one-to-four blood passages, 5 x 10^3 blood-form trypomastigotes from each sample were inoculated in groups of five mice. Parasitemia evaluation was done daily (except weekends) starting on the 4th day of infection (Brener, 1962) and was evaluated until at least 30 days post-infection. Curves were plotted using the mean of the five mice in each group.

Mortality - Mice were observed for a period of up to 120 days post-inoculation and mortality was registered along the parasitemia curve. The results were expressed as the accumulated percentage during the observation period.

Metacyclogenesis and growth in culture - Metacyclogenesis was evaluated in M16 medium (Chiari *et al.,* 1980) and LIT medium used as a control. Flagellates (1.5 x 10^7) obtained from the exponential growth phase in LIT medium were seeded for each sample. Growth was tracked through counts in a Newbauer hemocytometer and results were expressed as the number of parasites/ml of culture. Differentiation was evaluated as the percentage of trypomastigotes in random counts of 500 forms on Giemsa stained smears.

Chronic phase histopathologic pattern - Two mice per sample were used for the eight analyzed samples. Fragments from the heart, skeletal muscle, diaphragm, liver, spleen, esophagus and large and small intestine of mice that had been infected for seven months were analyzed. The tissue fragments were fixed in 10% formol and embedded in paraffin. Three sections of 3μm thickness spaced 20μm apart were processed on glass slides. After dying with hematoxilin-eosin, the parasite study was carried out by examining the entire section with 400X objective. The spleen sections were examined with immersion objective (100X). The number of inflammatory foci was determined by examination of 50 random microscopic fields of each section with 40X objective.

Results

Infectivity of culture forms and maintenance of samples - All samples were infective to mice except PR168 which in five experiments did not infect mice when assessed by fresh blood examination and hemocultures. To confirm that PR 168 strain is in fact *T. cruzi* additional experiments using RAPD and SSR - PCR DNA analysis revealed band pattern characteristic to *T. cruzi* species (data not shown). After the first inoculation, infection was detected by fresh blood examination (PP) in 27 samples and by hemoculture (SP) in three samples (PR 101, PR 150, PR 2052) (Table 1). Sixteen samples maintained by blood passages (BP) presented parasitemia peaks on days 10, 20 or 30. Seven samples were maintained by up to five alternate passages (AP) to adapt in mice and thereafter maintained by blood passages (Table 1). The remaining seven samples (PR 101, PR 114, PR 150, PR 328, PR 367, PR 387, PR 2052) were maintained by five alternate passages but did not produce parasitemia compatible with maintenance by blood passages (Table 1). It was not possible to determine parasitemia curves for these samples.

It is worthwhile to note that no circulating parasites were observed in animals infected with PR 150 strain. Infection was detected only by hemoculture that turned positive late, around the 30th day post-inoculation and the number of parasites was always very low. Culture forms of the parasite appeared in clumps and were rounded with short, almost inevident flagella. With maintenance, they became epimastigotes that were under than longer, with short flagella. These characteristics were completely different from those observed in the other samples.

Course of experimental infections - In the 16 samples maintained by blood passages, the prepatent period varied from 4 to 7 days and the patent period from 13 to 54 days. Four of the 16 strains had peak parasitemia of 10^3 parasites/ml and 12 had peak parasitemia between 10^4 and 10^6 parasites/ml of blood (Table 1 and Figure 1). The 7 samples that required alternate passages to adapt in mice and then were maintained by blood passages (AP/BP) had prepatent periods that varied between 4 to 12 days and patent periods that varied between 13 and 26

days. In this group four samples reached peak parasitemia of 10^3 parasites/ml and three samples had peaks of 10^4 parasites/ml of blood (Table 1 and Figure 1). Strains PR 182, PR 1055, PR 501 caused mortality (12,5%, 25,0 %, 20,0% respectively) in mice (Table 1). Based on these observations, the analyzed strains had low-to-medium virulence.

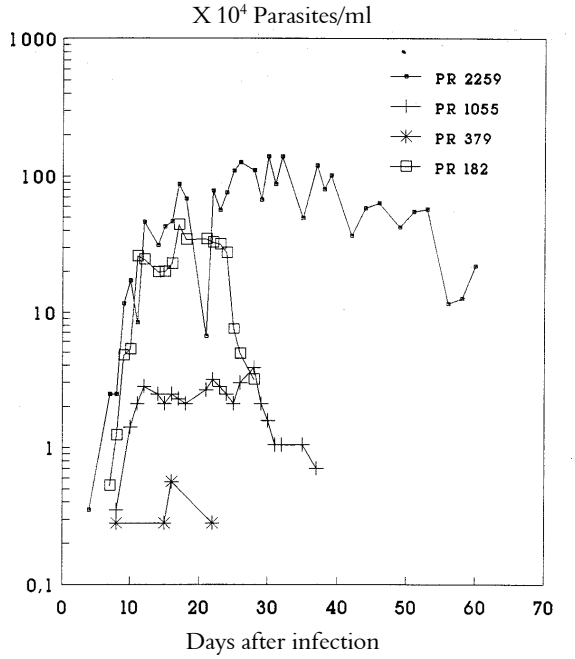

Figure 1. Parasitemia curves from C3H/He mice infected with 5000 blood trypomastigotes from selected *Trypanosoma cruzi* samples from chagasic patients from different geographic origins residing in the northwestern region of the state of Paraná

Metacyclogenesis and culture growth - These parameters were studied in 21 samples. High heterogeneity was observed in the metacyclogenesis rates with an accentuated tendency to produce low levels of epimastigote-trypomastigote transformation (Table 2). Of the 21 strains evaluated, 13 had differentiation between 2-27%; 5 had differentiation between 30-51%; and 3 had differentiation between 60-74% (Table 2). Parasite growth in LIT medium was in general higher than in M16 medium with low sample variation. Growth in both media was exponential from the beginning of the experiment until day 6, reaching a plateau on day 8.

Chronic phase histopathologic patterns - Eight samples were evaluated for tissue parasitemia in mice. Amastigote nests were observed in two samples, one in heart for PR 2259 strain and one in skeletal muscle for PR 182 strain (Table 3). Although parasites were observed only in these two tissues, inflammatory infiltrations of varying intensity and numbers were detected in these organs and also in liver and diaphragm (Table 3). Liver had the highest number of inflammatory foci (2-to-7/section) for five of the eight samples and perivasculite was also observed in this organ. Esophagus and large and small intestine fragments did not produce histopathologic pattern alterations in any of the samples and no parasites were seen in spleen sections.

Table 2. Metacyclogenesis and growth of different *Trypanosoma cruzi* samples in LIT and M16 medium

T. cruzi sample	Metacyclogenesis (%) 8th day of culture		Nº. of parasites[a] 8th day of culture	
	LIT	M16	LIT	M16
PR 036	1.6	2.0	44.8	29.3
PR 076	5.6	10.4	42.3	40.8
PR 149	3.8	74.0	113.8	67.5
PR 150	22.1	68.6	39.4	52.7
PR 168	8.2	35.4	73.0	37.5
PR 182	15.0	51.0	43.3	31.0
PR 184	1.0	4.6	81.5	54.5
PR 209	19.4	48.8	73.0	46.5
PR 316	1.4	2.2	36.8	17.3
PR 367	3.8	49.0	55.0	58.0
PR 379	0.8	3.6	60.5	41.2
PR 387	4.8	8.4	66.7	72.5
PR 396	1.3	5.6	51.0	36.2
PR 399	5.2	22.0	109.0	96.0
PR 401	1.2	10.6	112.0	80.0
PR 402	11.4	30.8	52.3	60.5
PR 427	1.6	4.2	101.8	60.0
PR 501	1.5	13.4	65.0	58.0
PR 1256	12.2	27.0	59.5	25.0
PR 1921	1.0	2.8	71.3	51.8
PR 2259	57.0	60.2	60.5	33.4

a: x 106parasites/ml

Discussion

This study demonstrated that samples of *T. cruzi* isolated from chronic chagasic patients from different geographic origins residing in the northwestern region of the state of Paraná had low and medium virulence with intraspecies variation. These findings were based on culture form infectivity, parasitemia curves, mortality, virulence, metacyclogenesis rates and histopathologic patterns in chronic infected mice.

Intraspecies variation is a well known phenomenon in *T. cruzi* populations that has been reported in studies of various parameters (Brener, 1979; Morel *et al.,* 1980; Romanha, 1982; Andrade, 1985). Since the 1950s (Herrer e Diaz, 1952/53) and later (Brener, 1979), authors have reported low-to-medium virulence in mice for the majority of strains obtained in nature in both wild and domestic cycles.

Several authors studied *T. cruzi* strains isolated from humans, triatomines and animals from different geographic regions (*Recôncavo Baiano, Paraíba, Piauí, Minas Gerais, Santa Catarina, Rio*

Grande do Sul, Amazônia, Chile) and reported that, in general, more than 80% of the strains presented low or medium virulence for mice when diverse biological parameters were considered (Andrade, 1973; Mello *et al.,* 1980; Schlemper, 1982; Carneiro *et al.,* 1991; Steindel, 1993; Fernandes, 1994; Coura *et al.,* 1995; Gomes *et al.,* 1995; Gonzalez *et al.,* 1995; Castro, 1997).

Table 3. Histopathology of organs of mice chronically infected with *Trypanosoma cruzi* isolated from patients from different geographic origins residing in the northwestern region of the state of Paraná

Sample	Heart	Muscle	Liver	Diaphragm
Control[a]	no changes	no changes	1 inflammatory infiltration	no changes
PR 182	4 inflammatory infiltrations	3 inflammatory infiltrations, 1 amastigote nest	4 inflammatory perivascular infiltrations	1 inflammatory infiltration
PR 209	no changes	no changes	7 inflammatory infiltrations	no changes
PR 1256	no changes	no changes	2-3 inflammatory infiltrations	no changes
PR 2259	1 inflammatory infiltration, 1 amastigote nest	3 inflammatory infiltrations	2 inflammatory infiltrations	diffuse inflammation
PR 036	1 inflammatory infiltration	1 inflammatory infiltration	5 inflammatory infiltrations	diffuse inflammation
PR 1291	no changes	no changes	no changes	no changes
PR 149	no changes	1 inflammatory infiltration	no changes	no changes
PR 076	3 inflammatory infiltrations	1 inflammatory infiltration	no changes	no changes

a: uninfected mice whose organs were processed identically to those of experimental animals were used as a control

Literature data indicate that there exists in nature a predominance of *T. cruzi* strains with low-to-medium virulence. These findings are not surprising since the elimination of the vertebrate host would endanger the maintenance and survival of the species, demonstrating the tendency to balance the parasite/host relationship. Gomes *et al.* (1995) further speculated that strains with low virulence and pathogenicity could protect against infections with more virulent strains. Absence of infectivity was also noted in the literature. Using parasites differentiated *in vitro* to infect irradiated nude Balb/c mice Sanches *et al.* (1990) observed lack of infectivity in 7 strains.

The *T. cruzi* strains studied here were isolated from chagasic patients from different endemic areas (58.6% from Minas Gerais, 19.4% from São Paulo, 12.9% from Paraná and 9.7% from various northeastern states (Table 1), who were residing in the northwestern region of the state of Paraná. The fact that this region is a migratory destination allowed us to conduct at the same time and under the same experimental conditions the isolation and study of the biological behavior of strains from different geographic origins. We observed that 48.4% (15) of the samples had medium virulence, 25.8% (8) had low virulence, 22.6% (7) did not produce patent parasitemia in infected mice and 3.2% (1) did not infect this animal. No high-virulence strain was isolated. Our results agree with the majority of the data in the literature. This homogeneous behavior could be related to the fact that all the samples were isolated from chronic chagasic patients. Very recent data obtained by Gomes *et al.* (1998) corroborate the existence of this homogeneity in strains isolated from chronic chagasic patients. Using a RAPD and SSR-PCR DNA analyses these authors showed that the majority of strains clearly formed a broad, genetically well correlated group. However, no correlation between biologic and molecular markers was observed. Further, based on biologic and molecular markers (Gomes *et al.,* 1998), the strains in the present study did not group according to geographic origin.

Wallace *et al.* (1995) reported that genetically distant *T. cruzi* strains presented distinct biological behavior in isogenic murine model. Here, the only exception among the *T. cruzi* samples was PR 150 strain, which had quite distinct biological behavior and also formed a genetically divergent group compared to the other strains (Gomes *et al.,* 1998).

As expected, the highest metacyclogenic percentages were observed in M16 medium (epimastigote→trypomastigote differentiation prompter), although the most abundant growth was registered in LIT medium. In general the samples that presented high metacyclogenesis in M16 also differentiated in LIT. Metacyclogenesis rates in M16 medium varied between 2.0-78.0%, while in LIT medium the variation was between 1.6-57.0% (Table 2). Sanchez *et al.* (1990) also reported a correlation between spontaneous metacyclogenesis capacity and special medium (TAU-3AAG). This supports reports that certain substances can stimulate epimastigote→trypomastigote differentiation (Fraidenraich *et al.,* 1993) as well as illustrates that metacyclogenesis is an intrinsic characteristic to each strain and that special medium only accentuates this characteristic (Chiari, 1981).

Regarding histopathologic evaluation, the observed inflammatory fibroid lesions were similar to those described by Andrade *et al.* (1989). In untreated, chronically infected mice, these authors observed inflammatory lesions and fibroids in heart and skeletal muscles with no parasite visualization, which confirms the difficulty in encountering

parasites in histologic sections of chronically infected mice. In the present study, besides the histologic alterations described above, the presence of the parasite was observed in two samples (PR 182 and PR 2259). These two samples were among those maintained by blood passages without the need for alternate passages for adaptation in mice, they presented high parasitemia levels and one caused 12.5% mortality in the inoculated animals (Table 1). If immunocitochemical techniques had been used (Barbosa, 1985), the chance of encountering the parasite in the other samples may have been higher.

Coura *et al.,* (1996) reported that regional differences in Chagas disease morbidity are probably related to a series of factors including the parasite, its vectors and its reservoirs. In the present work, we began the study of *T. cruzi* samples isolated from patients from various geographic regions residing in northwestern region of the state of Paraná. Since the number of samples from northwest Paraná was small, additional comparative studies with more human samples as well as isolates from triatomines and reservoirs should be carried out to better understand biological and molecular characteristics of *T. cruzi* populations that circulate in this region.

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