Morphological, biological and molecular characterization of three strains of *Trypanosoma cruzi* Chagas, 1909 (Kinetoplastida, Trypanosomatidae) isolated from *Triatoma sordida* (Stal) 1859 (Hemiptera, Reduviidae) and a domestic cat

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SUMMARY

A study was conducted of the biological, morphological and molecular characters of 3 strains of *Trypanosoma cruzi* (SI_5, SI_8 and $SIGR_3$) isolated from specimens of *Triatoma sordida* collected in Santo Inácio and a domestic cat. In order to carry out the study, the following parameters were evaluated: pre-patent period, parasitaemia curves, morphology of the parasites, mortality rates, histopathological lesions and molecular typing. The strains presented variable pre-patent periods, low parasitaemia and no animal mortality. The morphological study of trypomastigotes showed a predominance of intermediate-width and short-length forms, as well as low nuclear index. Epimastigotes presented a low nuclear index, intermediate-width forms in strains SI_5 and SI_8, and large-width forms in $SIGR_3$. A shorter length could be noted in strains SI_8 and $SIGR_3$, whereas SI_5 displayed an intermediate length. The histopathological study did not detect amastigote nests in tissues. The amplification of the divergent domain of 24Sα rRNA, HSP60 and GPI genes of strains SI_5, SI_8 and $SIGR_3$ classified the 3 strains into Group II. Biological parameters made it possible to classify the strains isolated in Santo Inácio (BA) into Biodeme III, Zymodeme 1 and Group II of *T. cruzi*.

Key words: *Trypanosoma cruzi*, Chagas' disease, characterization, biology, lineage, DNA, DTUs, histopathological, experimental infection, strains.

INTRODUCTION

Populations of *Trypanosoma cruzi*, which is the Chagas disease aetiological agent, have vast intraspecific variability, including differences related to morphology, virulence, pathogenicity, evasion ability in the case of an immune response from the host, sensitivity to medication, antigenic composition and biochemical properties (Fernandes *et al*. 1998; Tibayrenc and Ayala, 2002). It is possible that such diversity is associated with their adaptation and survival in different hosts (Zingales *et al*. 1997).

Data provided by the World Health Organization (WHO) indicate that 10 million people in Latin America have Chagas disease and 25 million live in risk areas (WHO, 2010). Different clinical manifestations of the disease in humans can be associated either with specific strains or with genetic markers of the host, although both can influence the course of the infection (Sturm *et al*. 2003).

Around 140 species of triatomines are potential vectors of *T. cruzi* (Rocha *et al*. 2009). In Brazil, there are 52 species, but only 5 of them – *Panstrongylus megistus*, *Triatoma brasiliensis*, *T. infestans*, *T. pseudomaculata* and *T. sordida* – have considerable epidemiological importance, as they colonize both in and around houses (Coura and Dias, 2009). In 1950, the vector-borne transmission of Chagas disease started to be controlled in the state of São Paulo, Brazil, by activities directed towards its main vector, *T. infestans*. That species, which is strictly domestic, was controlled in Brazil, Chile and Uruguay, and progress has been made to eradicate it in other countries of South America (Coura and Dias, 2009). On the other hand, in 2 Brazilian states, Bahia and Rio Grande do Sul, *T. infestans* can still be found

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in residences, although at low density (Moncayo and Silveira, 2009).

As soon as satisfactory results were achieved regarding the control of *T. infestans*, triatomines of secondary importance, collected in peridomestic areas, started gaining relevance. Among the triatomine species with higher epidemiological importance, *T. sordida* has been found within the state of São Paulo, in cities such as Ribeirão Preto, São José do Rio Preto and Araçatuba (Silva *et al*. 2003). Some authors have also reported the presence of *T. sordida* in areas within the state of Bahia, the city of Santo Inácio has being an example (Cerqueira *et al*. 1998). The presence of vector colonies in peridomestic areas can indicate an imminent colonization inside the houses if control measures are not continuously taken. There is no doubt that a careless attitude from residents and the lack of notification about the presence of such insects in the area are factors that can contribute to their proliferation (Silva *et al*. 2003).

This investigation was conducted to provide a biological, morphological and molecular characterization of *T. cruzi* isolated from specimens of *T. sordida* and a domestic cat in an urban community in the municipality of Santo Inácio (BA).

MATERIALS AND METHODS

Isolation of three strains of Trypanosoma cruzi

The strains SI_8 and SI_5 of *T. cruzi* were isolated from specimens of *T. sordida* collected in the urban community in Santo Inácio, Bahia by João Aristeu da Rosa, 2004 (*personal communication*). Strain $SIGR_3$ was isolated by xenodiagnosis on a cat from the same place by João Aristeu da Rosa, 2007 (*personal communication*). Since then the strains have been kept by successive passaging in Swiss mice and LIT (liver infusion tryptose) culture medium, showing exponential growth.

Trypanosoma cruzi *control samples used*

The strain Tm of *T. cruzi* was isolated from *Triatoma melanocephala* collected in the rural community in Poções, Bahia, 2009. The strain Tl was isolated from *Triatoma lenti* collected in the rural community in Macaúbas, Bahia, 2009. Both strains were isolated by abdominal compression of triatomines and have been kept in LIT culture medium.

Animals

For each strain, 5 isogenic male BALB/c mice aged around 30 days and weighing approximately 20 g were inoculated with blood trypomastigote forms by intraperitoneal injection. The procedure was performed using tuberculin syringes with a BD needle of 10 × 5 and a 0·3 ml dosage of blood collected from an infected mouse by cardiac puncture. The concentration of 5×10^3 blood trypomastigote forms per 0·3 ml of blood was adopted as standard.

Biological characterization

To study the parasitaemia curve, 15 animals were intraperitoneally inoculated with 5×10^3 trypomastigote forms of *T. cruzi*. In order to establish the infection pattern, 5 μl of blood obtained from the mice's tails were examined microscopically and the number of forms was established according to Brener's technique (1962).

Counts were performed in all the strains studied (SI_5, SI_8 and $SIGR_3$) on alternate days starting from the second day after the initial inoculation until the 60th day of the infection. The number of animals that could die during the course of the infection was observed in a daily basis.

The study of the dynamics of growth of epimastigote forms in all strains studied was carried out inoculating 5×10^6 parasites/ml in 5 ml of LIT medium. Counts were performed in triplicate for 10 days in a Neubauer chamber under an optical microscope.

Morphological characterization

The observation of blood trypomastigote forms required the use of blood smears obtained from the animals' tails during the acute phase. The methodology defined by Dias and Freitas Filho (1943) and Barreto (1965) was adopted as the morphological parameter.

The epimastigote forms studied were collected from the LIT culture on the 7th day after the initial inoculation, when the plateau phase of the growth curve was reached (Rossi, 2007). The smears were fixed with methanol, stained by the Giemsa method and then observed under an optical microscope at 1000× magnification. Thirty epimastigotes were studied and measured per strain, according to criteria described by Brener and Chiari (1963) to characterize slender, large and intermediate forms. The mean values obtained underwent Variance Analysis to verify if there were statistically significant differences among them within a significance level of 5%.

Histopathological study

For each strain, 21 infected animals were euthanized, 3 in each of the following days of the course of the infection: 7th, 10th, 14th, 20th and 30th, for the study of the acute stage, and 150th and 180th, for the study of the chronic phase. Fragments of the following organs were taken from the animals: heart, spleen, liver and skeletal muscle (thigh). Such fragments were kept in 10% formalin for 24 h. After being washed in running water, the pieces underwent dehydration and clearing so that they

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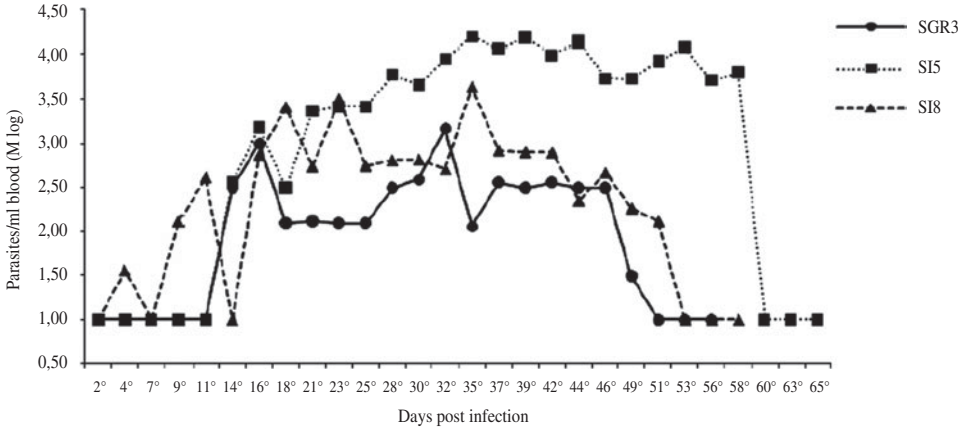

Fig. 1. Parasitaemia curves from three *Trypanosoma cruzi* populations in isogenic BALB/c mice, expressed as logarithmic mean (Mlog).

could be embedded in paraffin. Then 5-μm sections of each piece were cut with a microtome, placed on slides and stained with haematoxylin and eosin, according to the Ramos technique modified by Nai *et al*. (2004). The sections were examined under an optical microscope.

Molecular typing of T. cruzi *isolates*

DNA from culture trypanosomes ($\sim 1 \times 10^6$ parasites) was extracted using the phenol/chloroform method (Lewis *et al*. 2009). Genotyping the *T. cruzi* was done using PCRs based on LSU 24 Sα-rDNA (Souto *et al*. 1996), HSP60 and GPI (Westenberger *et al*. 2005) gene sequences. The genotyping assay was performed as published by Lewis *et al*. (2009).

PCR amplification of LSU 24 Sα-rDNA, HSP60 and GPI genes

The amplification process was performed in a thermal cycler (Gene Amp PCR System 9700 Applied BiosystemsTM) with the following thermal profile: 24 Sα rRNA gene, 94 °C for 5 min for enzyme activation and 30 cycles, including denaturation at 94 °C (30 sec), annealing at 55 °C (30 sec) and extension at 72 °C (30 sec), the final extension taking 7 min at 72 °C. The amplification process of the HSP60 and GPI genes was performed with the following thermal profile: 94 °C for 3 min for enzyme activation and 4 cycles, including denaturation at 94 °C (30 sec), annealing at 64 °C (30 sec) and extension at 72 °C (1 min). The process was repeated at 94 °C (30 sec) and for 28 cycles, including denaturation at 60 °C (30 sec), annealing at 72 °C (1 min) and extension at 72 °C (10 min).

PCR-RFLP analysis of HSP60 and GPI genes from T. cruzi

The primers and PCR conditions employed for amplification of HSP60 and GPI gene have been described previously (Westenberger *et al*. 2005; Lewis *et al*. 2009). Amplified HSP60 and GPI genes were digested with several restriction enzymes. The enzyme *EcoRV* was selected using the PCR-RFLP for the HSP60 gene and the enzyme *HhaI* was selected for the GPI gene. Length and restriction profiles of amplified HSP60 and GPI genes were analysed by electrophoresis in 1–3% agarose gels stained with ethidium bromide.

Ethics

All the procedures were reported to and approved by the Ethical Committee for Animal Experimentation at UNESP – Araraquara – SP (Protocol n. 12/2008).

RESULTS

Biological characterization

The *T. cruzi* isolates found in the Santo Inácio district showed low parasitaemia and infectivity, as can be seen in the average parasitaemia curve (Fig. 1). The pre-patent periods were relatively short: while SI_5 and $SIGR_3$ presented an 11-day period, the period for strain SI_8 varied between 2 and 21 days (Table 1).

The maximum parasitaemia for strain SI_5 was reached on the 35th day ($139{\cdot}95 \times 10^3$ forms), whereas for strains SI_8 and $SIGR_3$ it was reached on the 37th ($37{\cdot}32 \times 10^3$ forms) and 32nd ($30{\cdot}11 \times 10^3$ forms) days, respectively. After that period, the number of blood trypomastigotes starts to decrease, disappearing from the bloodstream around the 60th day of the infection.

The behaviour of each of the strains in mice showed a slow but regular development of parasitaemia until reaching a peak, followed by a regular decrease until the parasites disappeared from the peripheral blood. Animals inoculated with 5×10^3 trypomastigote forms of strains SI_8, SI_5 and $SIGR_3$ of *T. cruzi* showed an infection level of 80% for strains

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Table 1. Period pre-patent and patent, mortality, infectivity and prevalence in the blood of infected BALB/c mice in three *Trypanosoma cruzi* populations

Strain	Period (days)		Mortality %	Infectivity	Form Predominant
	Pre-patent	Patent			
$SIGR_3$	11	08–16	0	80%	Intermediate
SI_5	11	19–20	0	80%	Intermediate
SI_8	2–21	05–21	0	100%	Intermediate

Table 2. Mean morphometric parameters of blood trypomastigote forms and epimastigote forms of three *Trypanosoma cruzi* populations

Morphometric parameters	Mean values (μm) (trypomastigotes)			Mean values (μm) (epimastigotes)		
	SI_5	SI_8	$SIGR_3$	SI_5	SI_8	$SIGR_3$
Total length	19·15	16·95	19·02	28·56	21·96	26·17
Width	1·74	1·51	1·58	2·43	2·07	2·40
Nuclear index	0·64	0·62	0·70	0·34	0·27	0·36

SI_5 and $SIGR_3$ and 100% for strain SI_8. All the mice infected with the 3 strains survived and developed to the chronic stage. By 180 days after the mice had been inoculated, the infection with the 3 strains (SI_5, SI_8 and $SIGR_3$) had resulted in no deaths.

However, when their growth in LIT medium was represented in curves, some differences among the strains could be noted (Fig. 5). Strain SI_5 showed a growth phase on the 8th day ($8{\cdot}81 \times 10^6$ parasites), as well as strain $SIGR_3$ ($21{\cdot}79 \times 10^6$ parasites), whereas strain SI_8 showed a growth phase on the 7th day ($16{\cdot}07 \times 10^6$ parasites), as well as control strain Y ($13{\cdot}90 \times 10^6$ parasites). Together, these results suggest that the maintenance of populations of *T. cruzi* II may be related to intrinsic characteristics of the parasite, such as its infection ability.

Morphological characterization

By observing the blood trypomastigote forms of strains SI_5, SI_8 and $SIGR_3$ during the parasitaemia peak of the experimental infection by *T. cruzi*, it was possible to characterize them as follows: intermediate width ($P = 0{\cdot}0626$), short length ($P < 0{\cdot}0001$) and low nuclear index ($P = 0{\cdot}0577$) (Table 2).

Epimastigote forms showed: low nuclear index ($P < 0{\cdot}0001$) in all 3 strains; intermediate width in strains SI_5 and SI_8, and large width ($P = 0{\cdot}0014$) in strain $SIGR_3$; short length in strains SI_8 and $SIGR_3$, and intermediate length in strain SI_5 ($P < 0{\cdot}0001$) (Table 2).

Histopathological study

The sections prepared from heart, spleen, liver and skeletal muscle were examined under an optical microscope, but no tissue parasitism was observed, only mild inflammation was present (Fig. 6).

Molecular characterization

According to the DNA analysis by PCR amplification reaction, the strains isolated from specimens of *T. sordida* and a domestic cat in the urban community in Santo Inácio can be classified as belonging to *T. cruzi* group II. The amplified products presented 125 bp for the 24 Sα rRNA gene, 432–462 pb for the HSP60 gene, and 1264 bp for the GPI gene (Figs 2–4).

DISCUSSION

A distinctive character of *T. cruzi* is its pathogenicity towards vertebrate hosts, but the level of aggressiveness varies according to the strain under study. Therefore, some strains can be highly pathogenic while others are completely unaggressive (Andrade, 1974).

Many authors, including Barreto (1965), Belda Neto (1973) and Andrade (1974), mention the variation of parasitaemia in animals inoculated with *T. cruzi* strains, either for samples collected from humans, wild animals or the vector itself.

The 3 strains examined in this study showed relatively short pre-patent periods, a pattern that was also observed by Martins *et al*. (2008) while studying strains isolated from specimens of *Triatoma rubrovaria* collected in the Quaraí town, Rio Grande do Sul. That variation is frequently seen when a new strain of the parasite is isolated, and it is directly related to the stabilization of the relationship between the parasite and the vertebrate host (Albuquerque, 2001).

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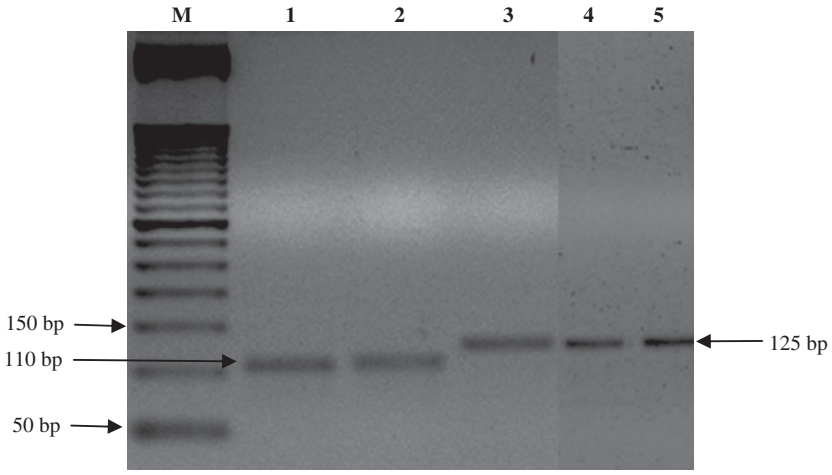

Fig. 2. DNA profiles generated by genotyping of isolates of *Trypanosoma cruzi* using PCR assays based on 24 S*a* rRNA. M, 50-bp molecular weight marker. Lane 1, Tm (control sample); lane 2, T lenti (control sample); lanes 3–5; SI_5; SI_8; $SIGR_3$ strains, respectively.

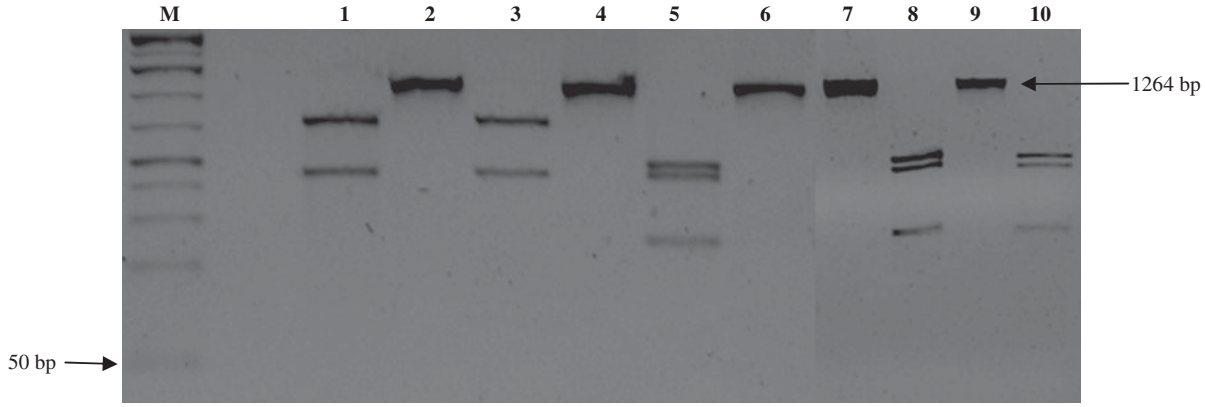

Fig. 3. DNA profiles generated by genotyping of isolates of *Trypanosoma cruzi* using PCR assays based on GPI fraction. M, 50-bp molecular weight marker. Lane 1, strain Tm (control sample/GPI gene digested); lane 2, strain Tm (GPI gene not digested); lane 3, T lenti, (control sample/GPI gene digested); lane 4, T lenti (GPI not digested); lane 5, SI_5 strain (GPI gene digested); lane 6, SI_5 strain (GPI gene not digested); lane 7, SI_8 strain (GPI gene not digested); lane 9, $SIGR_3$ strain (GPI gene not digested); lane 8, SI_8 strain (GPI gene digested); lane 10, $SIGR_3$ strain (GPI gene digested).

Inoculation of laboratory animals with *T. cruzi* strains has resulted in a variable mortality rate (Andrade, 1974; Magalhães *et al*. 1985; Barata *et al*. 1988). In this study, such rate was equal to zero for all strains. Mice were euthanized after 180 days of the infection and no trypomastigote forms were identified in their peripheral blood. The data obtained suggest that the characteristics found for a strain in the laboratory along the years would be the result of the behaviour interaction of many clones that, being favoured by certain handling conditions, had their expression conserved in a distinctive way in comparison to other sub-populations, as already pointed out by de Araújo and Chiari (1988).

The biological study (biodeme) revealed that the strains collected in Santo Inácio (SI_5, SI_8 e $SIGR_3$) belong to Biological Type III (Andrade, 1974) and Zimodeme 1 (Miles *et al*. 1980). The characteristics of that group include large and intermediate forms in the peripheral blood along the course of the infection, a parasitaemia peak around the 25th–30th days or after that period, and the low mortality rate of animals.

The trypomastigote form of *T. cruzi* can present morphological variations, as reported by Chagas back in 1909. The results obtained in this study agree with the literature, since large and intermediate blood trypomastigote forms have prevailed in most of the strains isolated from humans, triatomines and wild mammals (Brener and Chiari, 1963; Andrade, 1974; Pinto, 2000).

According to the hypothesis proposed by Zeledon and Vieto (1958), there would be a correlation between the nuclear index value and the virulence of a strain, whereupon the adaptation of a strain to a

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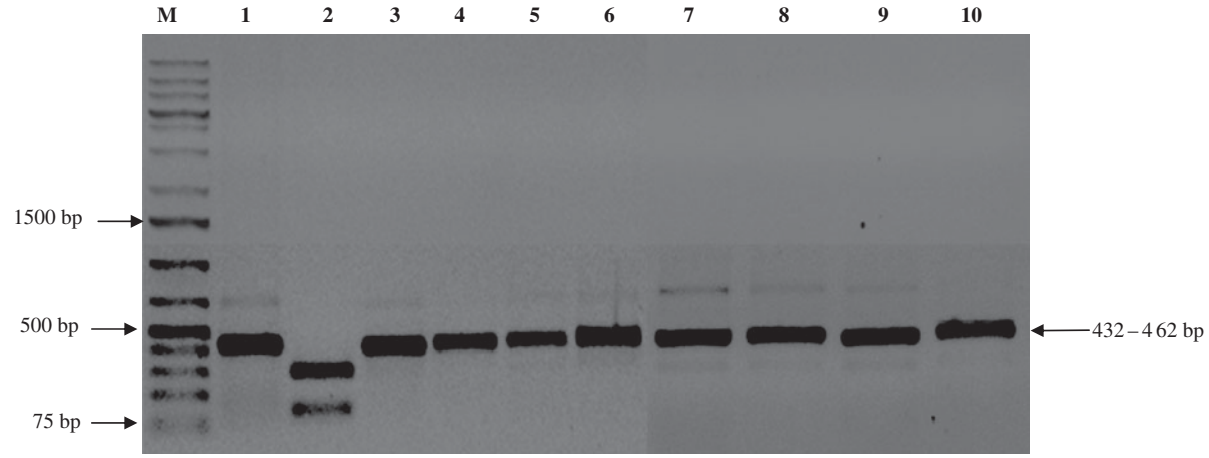

Fig. 4. DNA profiles generated by genotyping of isolates of *Trypanosoma cruzi* using PCR assays based on HSP60 gene. M, 1-Kb Plus molecular weight marker. Lanes 1 and 2, Tm strain (control sample, HSP60 gene not digested and digested); lanes 3 and 4, T lenti strain (control sample, HSP60 gene not digested and digested); lane 5, SI_5 strain (HSP60 gene not digested); lane 6, SI_5 strain (HSP60 gene digested); lanes, 7 and 8: SI_8 strain (HSP60 gene not digested); lanes, 9 and 10, $SIGR_3$ strain (HSP60 gene digested).

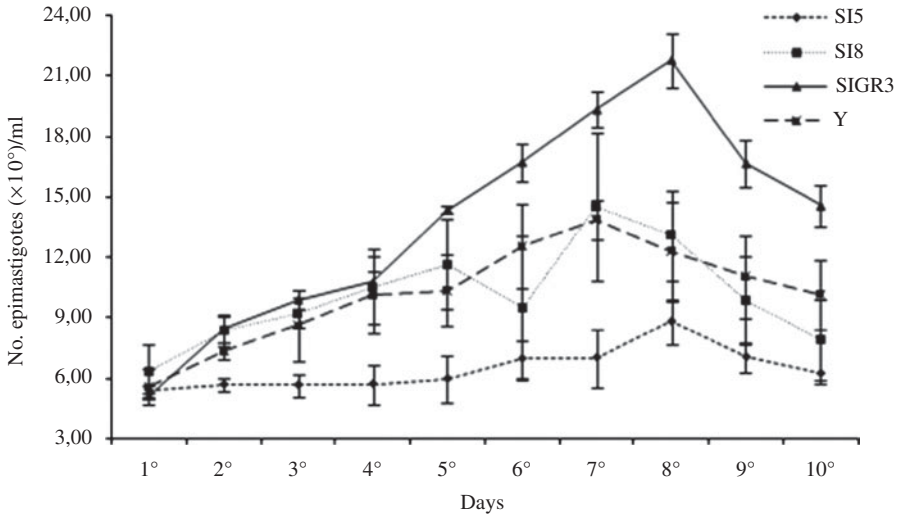

Fig. 5. Kinetics of growth of strains of *Trypanosoma cruzi*. Epimastigote forms from strains SI_5, SI_8, $SIGR_3$ and Y were grown in LIT medium for 10 days. The number of parasites was measured by counting in a Neubauer chamber under an optical microscope. The values represent the average of 1 experiment performed in triplicate.

particular host would be followed by a decrease in its aggressiveness and by a migration of the nucleum to the posterior part of the parasite, causing the decrease of the nuclear index. The results obtained in this study do not support such an hypothesis because strain SI_8 presented a lower nuclear index and a 100% infectivity rate for mice. The general meaning of that polymorphism has not been sufficiently investigated yet, whereupon it is unknown whether it expresses a different biological behaviour from the strains or just reflects the existence of a morphological 'complex', as in the case of what occurs with other trypanosomes (Brener and Chiari, 1963).

Brener and Chiari (1965) found differences in the '*in vitro*' behaviour of 4 strains isolated from human cases and 3 isolated from *Triatoma infestans* in LIT medium. Chiari (1974), studying the behaviour of strain Y in LIT medium, found that there was a growth phase which corresponded to a period of 4 days. The number of epimastigotes was developing in the late exponential phase and reached its growth in the stationary phase. Our results support the authors mentioned above, as there was a growth phase on the 7th day for strain SI_8 ($16{\cdot}07 \times 10^6$ parasites) and Y ($13{\cdot}90 \times 10^6$ parasites) and on the 8th day for strains SI_5 ($8{\cdot}81 \times 10^6$ parasites) and $SIGR_3$ ($21{\cdot}79 \times 10^6$ parasites).

The existence of *T. cruzi* strains with tropism for different tissues was reported by Melo and Brener in 1978. The study of the density of parasites in the organs of vertebrates may help to understand the pathogenesis of the disease because neuronal destruction and inflammatory processes may be directly influenced by local parasitism (Koeberle, 1968).

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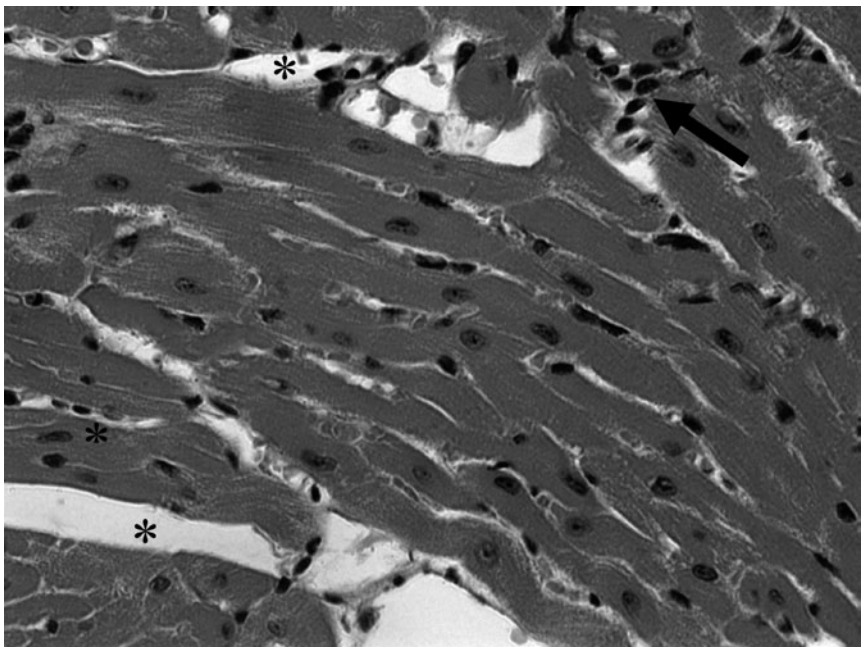

Fig. 6. Myocardium of mice necropsied 180 days after inoculation of *Trypanosoma cruzi* obtained from strain SI_5, in the presence of mononuclear cells (arrow) and intercellular spaces (*). Stain: Haematoxylin-Eosin. Magnification 20×.

However, tissue parasitism also depends on the intrinsic characteristics of the parasite, as well as the standardization of initial inoculation (Melo and Brener, 1978; Andrade and Magalhães, 1997; Martins *et al*. 2003). These authors used higher initial inoculation of 10 000 parasites/ml for histopathological study. In our study, we standardized the initial inoculum – 5×10^3/m (5000) – because the strains had low parasitaemia. Thus, it was not possible to view the parasite in tissues, which may have been caused by the reduced number of parasites, inherent characteristics of the strains, or the control of the experimental infection of BALB/c mice.

The presence of inflammatory changes is consistent with the observations of Andrade (1985), who noted that the parasites were often not located in tissues, particularly not in organs of animals or individuals with Chagas disease in the chronic phase. Observations by Carlos Chagas in the early twentieth century (1909) remain in effect and relate the presence of *T. cruzi* in tissue with the pathophysiological development of the disease. On the other hand, such observations reveal that it is common for a large number of individuals in the chronic phase to show abnormalities in tissues without the presence of the aetiological agent (Chagas and Villela, 1922).

Recently, based on the analysis of portions that codify the 24S*α* rRNA, GPI and HSP60 genes by means of PCR reaction, *T. cruzi* strains were divided into 6 groups (Lewis *et al*. 2009; Zingales *et al*. 2009). The DNA analyses of strains collected in Santo Inácio put them into *T. cruzi* group II, the same group of strain Y, even though they show notable behavioural differences during the experimental infection of mice, and thus belong to different Biodemes. Regardless of the fact that the strains were isolated from hosts at different times (SI_5 and SI_8 were isolated from specimens of *T. sordida* in 2003, whereas the isolation of $SIGR_3$ was performed by xenodiagnosis on a cat in 2006), the DNA analysis indicates that in that place there are strains belonging to the same *T. cruzi* group II, according to their molecular classification.

The characterization of strains SI_5 and SI_8 of *T. cruzi* – which were isolated together with another 10 from 18 specimens of *T. sordida* collected in the urban community in Santo Inácio, Bahia – showed no pathogenicity towards BALB/c mice regarding the morphometric parameters. Moreover, they were classified into Biodeme III (Andrade, 1974) and Zimodeme 1 (Miles *et al*. 1980), Group II (Zingales *et al*. 2009 and Lewis *et al*. 2009) and *T. cruzi* I (Anonymous, Satellite Meeting, 1999). All these characteristics are shared by strain $SIGR_3$, which was isolated in the same place by xenodiagnosis on a domestic cat.

The study of strains SI_5, SI_8 and $SIGR_3$ of *T. cruzi*, isolated from specimens of *T. sordida* and a domestic cat in the urban community in the district of Santo Inácio, Bahia, reinforces the need for entomological surveillance in Northeastern Brazil.

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