Characterization of *Trypanosoma cruzi* Strains Isolated from Chronic Chagasic Patients, Triatomines and Opossums Naturally Infected from the State of Rio Grande do Sul, Brazil

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Thirty-five Trypanosoma cruzi *strains were isolated from chronic chagasic patients, triatomines and opossums from different municipalities of the State of Rio Grande do Sul. Parasites were characterized by means of mice infectivity, enzyme electrophoresis and randomly amplified polymorphic DNA (RAPD) analysis. Twenty-nine strains were isolated from chagasic patients, 4 from triatomines (2 from* Triatoma infestans *and 2 from* Panstrongylus megistus*) and 2 from opossums* Didelphis albiventris. *Thirty-three* T. cruzi *strains were of low and 2 strains of high virulence in mice. Both virulent strains were isolated from* P. megistus. *Isoenzyme analysis of the strains showed 3 different zymodemes. Eleven strains isolated from chagasic patients and 2 from* D. albiventris *were Z2. Eighteen strains from patients and 2 from* T. infestans *were ZB and 2* T. cruzi *strains isolated from* P. megistus *were Z1. RAPD profiles obtained with 4 random primers showed a high genetic heterogeneity of the* T. cruzi *strains. Zymodeme 2 and ZB strains were the more polymorphic. A band sharing analysis of the RAPD profiles of Z2 and ZB strains using 3 primers, showed a very low percentage of shared bands, 20% among 13 ZB strains and 14% among 13 Z2 strains. According to the isoenzyme results, 3* T. cruzi *populations were present in State of Rio Grande do Sul. Zymodeme 2 and ZB strains were found infecting man (domiciliar transmission cycle) whereas Z1 strains were found infecting the sylvatic vector* P. megistus.

Key words: Chagas' disease - *Trypanosoma cruzi* - randomly amplified polymorphic DNA - isoenzyme characterization - epidemiology

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Trypanosoma cruzi, the ethiological agent of Chagas' disease is a digenetic flagellate protozoan transmitted to the mammal host by means of infected feces or urine of triatomine bugs (WHO 1991). In Brazil the endemic area of Chagas' disease comprises more than 3 million km^2, and it is estimated that around 5 million people are infected by *T. cruzi* (Dias 1987). Intraspecific variations in *T. cruzi* have been demonstrated at the biological, immunological, biochemical and genetic levels (Brener 1977, Miles et al. 1977, Morel et al. 1980, Andrade 1985, Tibayrenc & Ayala 1988, Steindel et al. 1993). Enzyme electrophoresis studies have demonstrated that distinct *T. cruzi* populations (zymodemes) are circulating in the domestic and sylvatic transmission cycles (Miles et al. 1977, 1978). Therefore, isoenzyme provides a good marker in epidemiological studies of Chagas' disease. Recently, randomly amplified polymorphic DNA (RAPD), has been used in studies of genetic diversity of trypanosomes (Dirie et al. 1993, Steindel et al. 1993, Tibayrenc et al. 1993). This technique has been widely applied to the study of parasite populations because of its simplicity, the lack of requirement of DNA sequence information for primer design and its great power in mapping genomes (Steindel et al. 1993). Studies of genetic variability using molecular markers such as kDNA (schizodemes) (Morel et al. 1980), DNA fingerprinting using multilocal probes (Macedo et al. 1992) and RAPD defined highly variable groups of *T. cruzi*. Recently, 2 independent nuclear markers, ribosomal RNA and mini-exon genes, allowed

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the division of *T. cruzi* strains into 2 groups (Souto et al. 1996).

In the Southern region of Brazil, the State of Rio Grande do Sul is the most important endemic area of human Chagas' disease with infection rates varying from 17.6% to 19.6% in the south region of the State (Baruffa & Alcântara Filho 1977, 1985). Investigation of the triatomine fauna showed that at least, 7 triatomine species were present. *Triatoma infestans* was the only domestic vector species found (Coutinho et al. 1952). According to the 1980 population census, it is estimated that around 700 thousand people are infected by *T. cruzi* in Rio Grande do Sul (Baruffa 1987). Although with such a high prevalence, a few number of *T. cruzi* strains have been studied and little is known about the parasite populations that circulate in the different transmission cycles there. In the present work, we characterized by means of virulence in mice, isoenzyme electrophoresis and RAPD analysis, 35 *T. cruzi* strains isolated from chronic chagasic patients, triatomines and opossums from Rio Grande do Sul.

MATERIALS AND METHODS

Parasite isolation - Authoctonous chronic chagasic patients from Rio Grande do Sul with positive immunofluorescence and/or hemagglutination antibody test, were submitted to xenodiagnosis with 30 fourth instar nymphs of *T. infestans* or hemoculture in liver infusion tryptose (LIT) medium for parasite isolation. Adults of *T. infestans* captured inside houses and *Panstrongylus megistus* collected in the peridomiciliar environment by personnel of the National Foundation of Health (FNS), were examined by fresh feces smears and the positive insects were submitted to a xenoculture in LIT for parasite isolation (Bronfen et al. 1989). Opossums *Didelphis albiventris* captured in peridomiciliar areas of Porto Alegre city by the Vector Control Division of the Health and Environment Secretary, were submitted to xenodiagnosis for searching and isolation of *T. cruzi*. The parasites isolated were maintained by weekly passages in LIT medium at 28^oC or cryopreserved in liquid nitrogen.

Parasitemia - Groups of 5 male albino mice weighting 15 to 18 g were inoculated with $5x10^3$ blood trypomastigotes by the intraperitoneal route with different *T. cruzi* strains. The parasitemia was followed every 2 days from the 6th to the 50th day of infection by fresh blood smears and the number of parasites estimated as described by Brener (1962). Curves were plotted using the mean parasitemia of 5 mice for each day of study. Mortality rate was expressed as percentage of accumulated deaths at the 60 day of inoculation. The virulence of the strains was determined according to prepatent period, parasitemia level and mortality rate.

Isoenzyme profile - Parasites were grown in flasks containing 30 ml of LIT medium at 28^oC, harvested in the exponential phase and washed 3 times by centrifugation, 2,000 x g, 4^oC, 10 min in KRT (Krebs Ringer Tris, pH 7.4) buffer and the parasite pellet was stored at -70^oC until use. The pellet was thawed at room temperature and submitted to osmotic lysis in an enzyme stabilizer (2.0 mM dithiothreitol, 2.0 mM ε-amino caproic acid, 2.0 mM Na_2 EDTA, pH 7.4) at 4^oC at a volume ratio 1:1. The lysate was centrifuged at 15,000 x g for 1 hr at 4^oC, and the supernatant (enzymatic extract) was collected and stored as 15 µl beads in liquid nitrogen. The following 6 enzymes were analyzed: alanine aminotransferase (ALAT) [E.C.2.6.1.2]; aspartate aminotransferase (ASAT) [E.C.2.6.1.1]; glucose phosphate isomerase (GPI) [E.C.5.3.1.9]; phosphoglucomutase (PGM) [E.C.2.7.5.1]; glucose-6-phosphate dehydrogenase (G6PD) [E.C.1.1.1.49] and malic enzyme (ME) [E.C.1.1.1.40]. Electrophoretic separation and enzyme staining were done as described by Carneiro et al. (1990). Standard *T. cruzi* zymodemes Z1, Z2, ZB and ZC were used as reference.

DNA preparation -The isolation, amplification and electrophoresis of the parasite DNA was essentially the same as described by Steindel et al. (1993). Briefly, parasite pellet was resuspended in 50 mM Tris-HCl/ 50 mM EDTA/ 100 mM NaCl/ 0.5% SDS, pH 8.0 (extraction buffer) and incubated with 20 $\mu g\ ml^{-1}$ proteinase K for 2 hr at 45^oC. Following phenol/chloroform extraction and ethanol precipitation the DNA was resuspended in 10 mM Tris-HCl/ 1 mM EDTA pH 8.0 (TE buffer) and digested with 1 U ml^{-1} RNAse (Sigma Chemical Co., St. Louis, Mo, USA) for 2hr at 37^oC. Following a new round of phenol/chloroform extraction and ethanol precipitation the DNA was resuspended in TE pH 8.0 and its concentration determined by comparison against known standard following agarose gel electrophoresis stained with ethidium bromide.

RAPD - The amplification reaction was done in a termocycler (MJR Research Inc. - PTC 100), in a final volume of 10 µl containing 0.8 units of *Taq* DNA polymerase (CENBIOT, RS, Brasil), 200 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.5 together with 6.4 *pmol* of each primer and 1.0 ng of template DNA.

The primers 3303 (5'- TCACGATGCA -3'), 3307 (5'- AGTGCTACGC -3'), λgt11F (5'- GGTG GCGACGACTCCTGGAGCCCG -3') and λgt11R (5'-TTGACACCAGACCAACTGGTAATG -3'), were arbitrarily selected from the laboratory stocks. The reaction mixture was overlaid with 20 µl of

mineral oil and, following an initial denaturation at 95°C for 5 min, it was subjected to 2 cycles through the following temperature profile: 30°C for 2 min for annealing, 72°C for 1 min for extension and 30 sec at 95°C for denaturation followed by 33 cycles where the annealing temperature was altered to 40°C. In the final cycle the extension step was for 5 min. After amplification, 3 µl of each reaction was mixed with 0.6 µl of 6x DNA sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and subjected to electrophoresis through a 4% non-denaturating polyacrylamide gel. Gels were fixed with 10% ethanol/ 0.5% acetic acid for 20 min and DNA bands revealed by staining with 0.2% silver nitrate for 30 min and reduction with 0.75 M NaOH/ 0.1 M formaldehyde for 10 min as described by Santos et al. (1993). The gels were analyzed by means of band sharing between all possible pairs and the percentage of common bands was determined.

RESULTS

T. cruzi isolation - From August 1992 to July 1993, xenodiagnosis and/or hemoculture were performed in 236 chronic chagasic patients from 42 municipalities of Rio Grande do Sul (Fig. 1). From the 29 *T. cruzi* strains isolated from chagasic patients, 18 were obtained by hemoculture an 11 by xenodiagnosis.Three hundred and forty three triatomines (231 *T. infestans* and 112 *P. megistus*) and 11 opossums were captured and searched for *T. cruzi*. Ten *T. infestans* (4.3%), 4 *P. megistus* (3.6%) and 2 opossums (18.2%) were found infected by *T. cruzi*. Only 2 strains from *T. infestans* and 2 from *P. megistus* were isolated by xenoculture. In addition, 2 strains were isolated by xenodiagnosis from the infected opossums (*D. albiventris*), captured in the peridomiciliar environment at Porto Alegre city (Table I).

Parasitemia profile - The 35 *T. cruzi* strains isolated produced patent parasitemia in experimentally inoculated albino mice and showed two distinct parasitemia profiles (Fig. 2). Two strains (RS11and RS21) isolated from the vector *P. megistus* were of high virulence for mice presenting a short prepatent period ranging from 6 to 8 days, displaying a rapid raising parasitemia reaching 13.1 x 10^3 parasites/ 5 mm^3 of blood and causing 100% mortality in 16 days. The remaining 33 strains were all of low virulence for mice with prepatent period of 12 to 18 days, and a peak of parasitemia of 900 parasites/ 5 mm^3 of blood around the 28th to 32nd day of infection and mortality rate varying from 0 to 10% at the 60th day of infection.

Isoenzyme profiles - Analysis of 6 enzymes of 35 *T. cruzi* strains from Rio Grande do Sul revealed

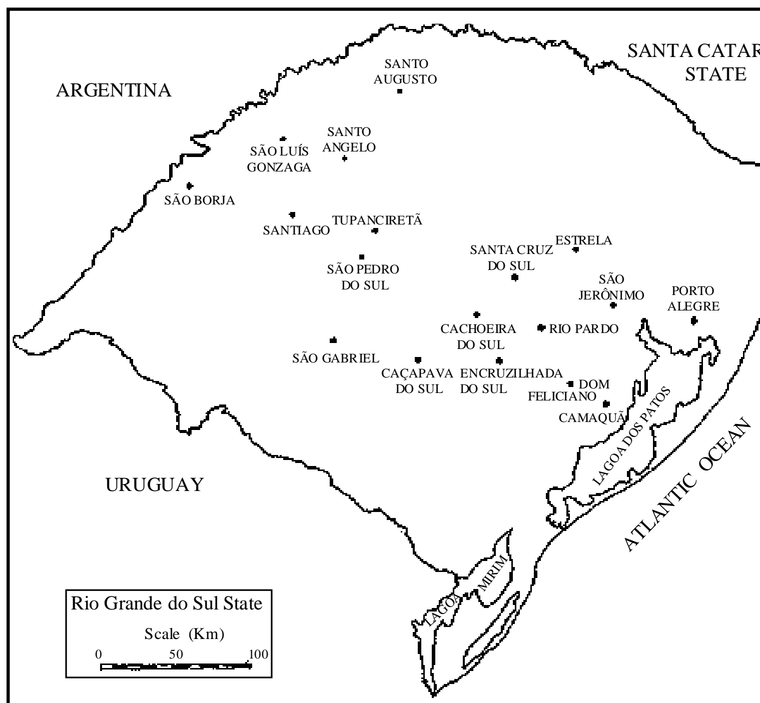

Fig. 1: map of the State of Rio Grande do Sul showing the location of municipalities where *Trypanosoma cruzi* strains were obtained.

TABLE I

Host, isolation methods and origin of *Trypanosoma cruzi* strains from the State of Rio Grande do Sul

Strains	Host	Isolation/method	Origin
RS2	Man	Hemoculture	Encruzilhada do Sul
RS8, RS12, RS13	Man	Xenodiagnosis	Encruzilhada do Sul
RS1, RS23	Man	Hemoculture	São Jerônimo
RS4, RS17	Man	Hemoculture	Caçapava do Sul
RS5, RS20, RS25, RS35	Man	Hemoculture	Santo Angelo
RS6, RS24	Man	Hemoculture	Rio Pardo
RS26	Man	Xenodiagnosis	Rio Pardo
RS7, RS27	Man	Xenodiagnosis	São Gabriel
RS16	Man	Hemoculture	São Gabriel
RS14	Man	Xenodiagnosis	São Pedro do Sul
RS18	Man	Hemoculture	Estrela
RS15	Man	Hemoculture	Santiago
RS19	Man	Hemoculture	Santo Augusto
RS22	Man	Hemoculture	São Luiz
RS28	Man	Xenodiagnosis	São Borja
RS33	Man	Hemoculture	Santa Cruz do Sul
RS36	Man	Hemoculture	Tupanciretã
RS30	Man	Hemoculture	Camaquã
RS29	Man	Xenodiagnosis	Porto Alegre
RS31	Man	Xenodiagnosis	Cachoeira do Sul
RS32	*Triatoma infestans*	Xenoculture	Cachoeira do Sul
RS34	*Triatoma infestans*	Xenoculture	Dom Feliciano
RS11, RS21	*Panstrongylus megistus*	Xenoculture	Porto Alegre
RS9, RS10	*Didelphis albiventris*	xenodiagnosis	Porto Alegre

3 major zymodemes Z1, Z2 and ZB (Fig. 3, Table II). In all zymodemes minor enzymatic variations were present. From the 29 strains isolated from chronic chagasic patients 11 were zymodeme 2 and 18 zymodeme B. Eight of the Z2 strains, represented by RS 5 strain in Fig. 3, displayed a double instead of a single band in the ASAT enzyme, and were called Z2a (Table II). Another Z2 strain (RS 7) presented an ASAT profile identical to the zymodeme C and was called Z2b. 2 strains from man (RS 4 and RS 36) and two from *D. albiventris* (RS 9 and RS 10) presented a typical zymodeme Z2. Sixteen strains from chagasic patients and 2 from *T. infestans* presented a typical zymodeme ZB. The ZB strains RS 25 and RS 33 presented a faster migration on the GPI's triplet bands. Additionally, RS 33 presented double band on ASAT and was called ZBa whereas, RS 25 presented a single band and was called ZBb. The strains RS 11 and RS 21, isolated from *P. megistus*, were identical to the reference Z1 strain in 5 out of 6 enzymes. They differed on ALAT enzyme and were called Z1a.

RAPD profiles - The RAPD profiles obtained with primer 3303 of 2 *T. cruzi* strains representative of each zymodeme found in Rio Grande do Sul together with Z1, Z2 and ZB reference zymodemes showed that the major groups defined by RAPD

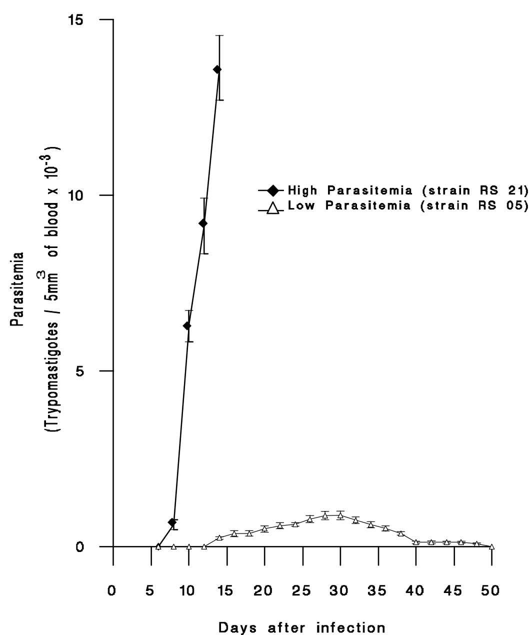

Fig. 2: representative parasitemia curves from *Trypanosoma cruzi* strains from the State of Rio Grande do Sul in albino mice inoculated intraperitoneally with $5x10^3$ blood trypomastigotes.

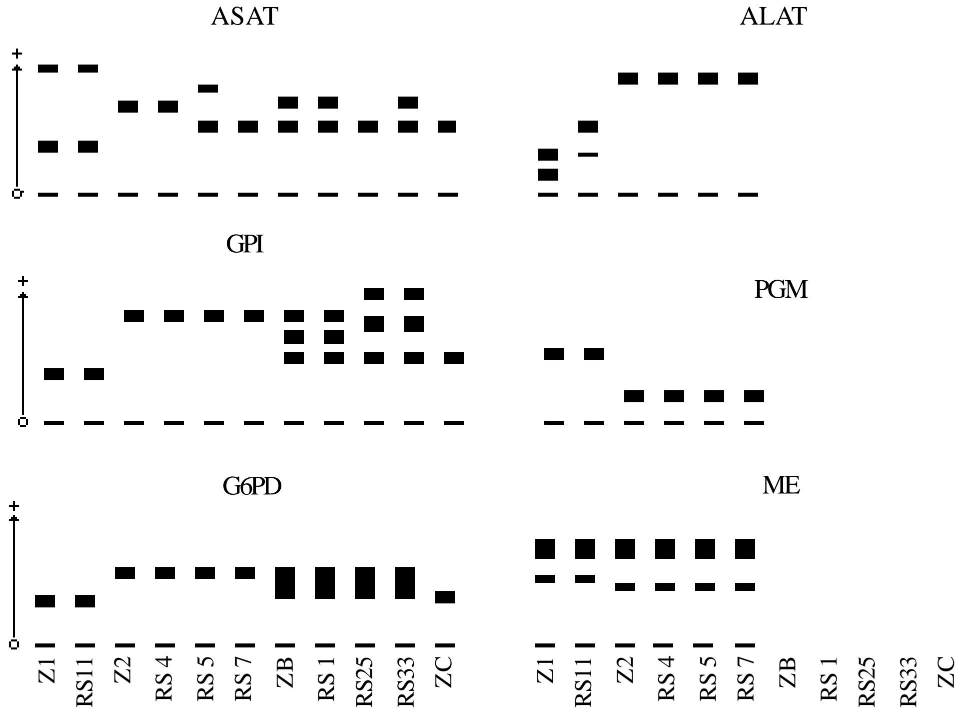

Fig. 3: diagrammatic representation of the isoenzymatic profiles of six enzymes from *Trypanosoma cruzi* strains from the State of Rio Grande do Sul. For enzyme and strain abbreviations see Materials and Methods and Table II.

TABLE II

Hosts and zymodemes of *Trypanosoma cruzi* strains from the State of Rio Grande do Sul

Strains	Host	Zymodeme	Variant enzymes
RS11, RS21	*Panstrongylus megistus*	Z1a (02)	ALAT
RS9, RS10	*Didelphis albiventris*	Z2 (02)	
RS4, RS36	Man	Z2 (02)	
RS5, RS6, RS26, RS27, RS28, RS29, RS30, RS31	Man	Z2a (08)	ASAT
RS7	Man	Z2b (01)	ASAT
RS1, RS2, RS8, RS12, RS13, RS14, RS15 ,RS16, RS17, RS18, RS19, RS20, RS22, RS23, RS24, RS35	Man	ZB (16)	
RS33	Man	ZBa (01)	GPI
RS25	Man	ZBb (01)	ASAT and GPI
RS32, RS34	*Triatoma infestans*	ZB (02)	
	Total	35	

In parenthesis the number of strains with that zymodeme

coincide with the same groups defined by isoenzyme (Fig. 4). A comparative study of RAPD bands of the 2 Z1 strains obtained with 3307 and λgt11R primers shows that from the 39 bands considered, 74% were present in both strains (data not shown). In contrast with the homogeneity of the Z1 strains, the Z2 and ZB *T. cruzi* strains presented highly polymorphic RAPD profiles with all primers used. Figs 5 and 6 illustrate the RAPD profiles of Z2 and ZB strains with primers 3307 and λgt11R respectively. A band sharing analysis using only primer λgt11F shows that the percentage of bands shared between 13 ZB strains was of 10/23 (43.4%) and of 7/21 (33.3%) between 13 Z2 strains. However, when

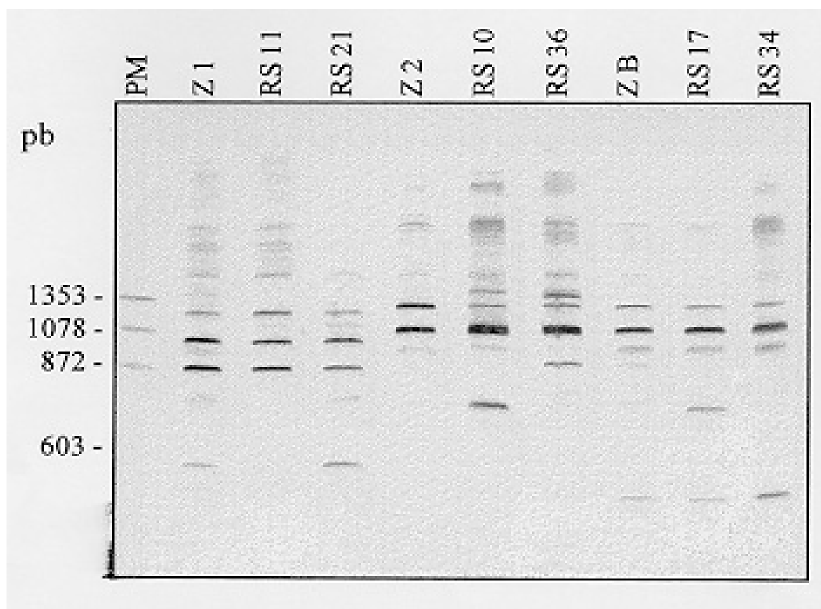

Fig. 4: a 4% polyacrylamide gel stained with silver shows the RAPD profiles of *Trypanosoma cruzi* strains from the State of Rio Grande do Sul together with standard *T. cruzi* zymodemes amplified with primer 3303. RS11 and RS 21 (Z1), RS 10 and RS 36 (Z2), RS 17 and RS 34 (ZB) are representative zymodemes from the State of Rio Grande do Sul. Z1, Z2 and ZB are the standard *T. cruzi* zymodemes.

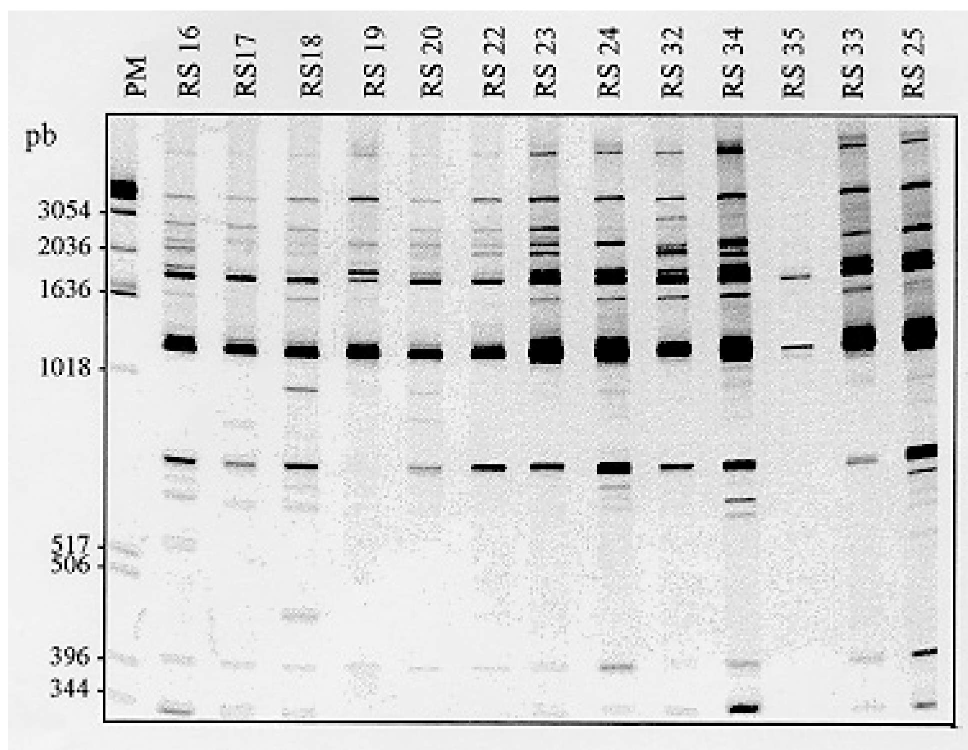

Fig. 5: a 4% polyacrylamide gel stained with silver shows the RAPD profiles of 13 zymodeme ZB *Trypanosoma cruzi* strains from the State of Rio Grande do Sul amplified with primer 3307.

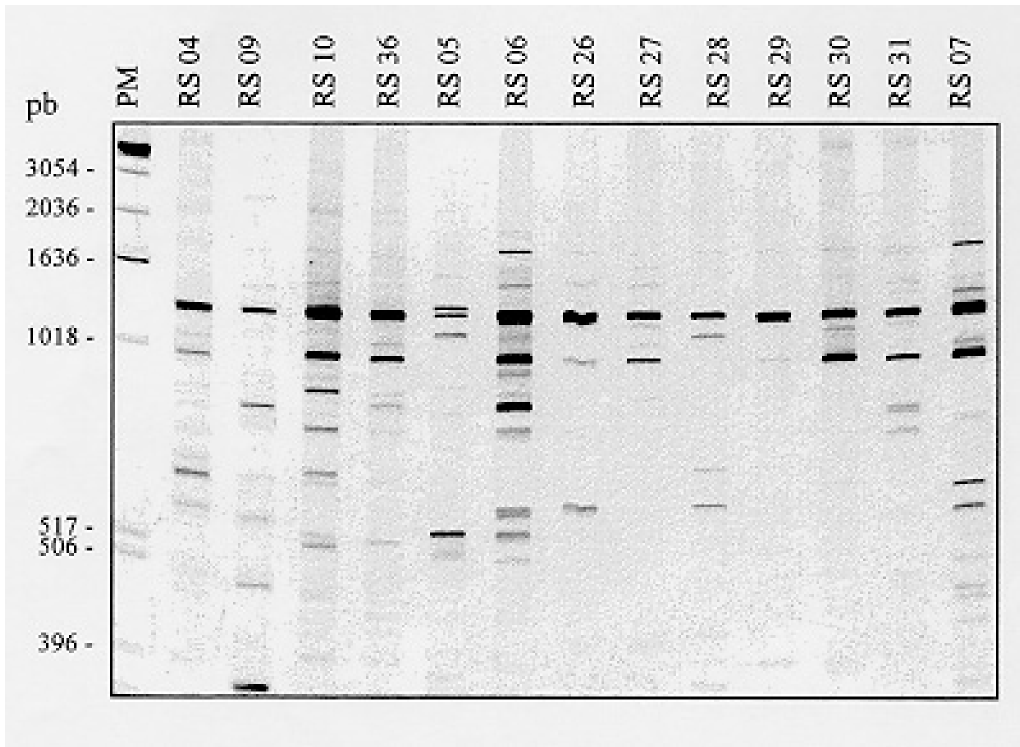

Fig. 6: a 4% polyacrylamide gel stained with silver shows the RAPD profiles of 13 zymodeme Z2 *Trypanosoma cruzi* strains from the State of Rio Grande do Sul amplified with primer λgt11R.

3 primers (3307, λgt11R and λgt11F) were used for a comparison of the RAPD profiles of the same ZB and Z2 strains, the percentage of bands shared within zymodeme ZB and Z2 strains was of 19/95 (20%) and 12/87 (14%) respectively.

DISCUSSION

T. cruzi populations have been characterized on the basis of biological behaviour in mice (Brener 1977, Andrade 1985). Parasitemia studies in albino mice of 35 *T. cruzi* strains isolated from chronic chagasic patients, and naturally infected vectors (*T. infestans* and *P. megistus*) and from opossums (*D. albiventris*) from Rio Grande do Sul, showed that 33 (94%) of the strains were of low and 2 (6%) of high virulence to mice. All 29 strains isolated from chronic chagasic patients as well as 2 strains isolated from *T. infestans* and 2 from *D. albiventris* induced similar parasitemia levels and were of low virulence to mice. On the other hand, strains (RS11 and RS21) isolated from *P. megistus* were highly virulent to mice. *T. cruzi* of high, medium and low virulence and strains that did not induce patent parasitemia in mice have been isolated from chronic chagasic patients (Schlemper Jr et al. 1983, Carneiro et al. 1991) and from sylvatic reservoirs and vectors (Steindel 1993). Our results confirm the predominance of *T. cruzi* strains of low virulence to mice in chronic chagasic patients.

Isoenzyme electrophoresis of 35 *T. cruzi* strains from Rio Grande do Sul showed the presence of 3 major zymodemes Z1, Z2 and ZB. *T. cruzi* Z1 was found associated with the sylvatic vector *P. megistus*, whereas Z2 parasites came from chronic patients and from opossums. Zymodeme ZB parasites were found in both chronic chagasic patients and in the domestic vector *T. infestans*. Our results show that in Rio Grande do Sul 2 independent transmission cycles of *T. cruzi* occur: a sylvatic cycle comprising Z1 parasites and the vector *P. megistus* and possibly wild mammals and a domestic cycle comprising *T. cruzi* Z2 and ZB, the vector *T. infestans* and man. *T. infestans* is the main vector of Chagas' disease and the only domiciliated triatomine species recorded in Rio Grande do Sul (Coutinho et al. 1952). Nowadays, due to the triatomine control campaign developed by the FNS, house infestation by *T. infestans* is rare. On the other hand, human dwellings are sporadically invaded by adults of wide spread sylvatic triatomine species as *P. megistus* and *T. rubrovaria*. The role of these triatomine species in *T. cruzi* transmission to

man is still unknown.

In Brazil, 6 major *T. cruzi* zymodemes (Z1, Z2, Z3, ZB, ZC and ZD) have been recorded from chagasic patients (Miles et al. 1977, 1978, Romanha 1982). In the central region of Brazil, zymodeme Z2 parasites are the most frequently found in chronic chagasic patients, whereas both Z1 and Z2 zymodemes were found in acute infections (Luquetti et al. 1986). *T. cruzi* Z2 (59.4%) and ZB (20.3%) were also the most frequent parasites isolated from chronic chagasic patients from Bambuí city, State of Minas Gerais (Romanha 1982). In the present work, 38% of the *T. cruzi* strains isolated from chagasic patients were Z2 and 62% were ZB. Our results show that in Rio Grande do Sul, at least 2 distinct *T. cruzi* zymodemes infect man. Isoenzyme data of *T. cruzi* strains from human and non human origin comprising a wide geographical distribution showed that this parasite presented a clonal structure (Tibayrenc & Ayala 1988, Ayala 1993). On the other hand k-DNA and DNA fingerprinting analyses show that a single *T. cruzi* strain may be composed by clones with different genetic characteristics (Morel et al. 1980, Macedo et al. 1992). The intrazymodeme heterogeneity found here in some *T. cruzi* strains from human and sylvatic origin suggests that different sub populations of the parasite may be present in the same host.

Opossums are the most important sylvatic reservoirs of *T. cruzi* known and play an important role in the maintenance of the sylvatic *T. cruzi* transmission cycle. Zymodeme Z1 parasites was the only zymodeme found in a large number of *T. cruzi* strains isolated from *D. albiventris* from Bambuí, Minas Gerais and from *D. marsupialis* from the State of Santa Catarina, respectively, endemic and non endemic areas of Chagas' disease in Brazil (Fernandes et al. 1991, Steindel 1993). In contrast, *T. cruzi* zymodeme Z2 was isolated from 2 naturally infected *D. albiventris* from Rio Grande do Sul. Since the opossums were captured in the peridomiciliar environment, they might have been infected by domestic vectors. The sinantropic behaviour of *Didelphis* sp., represents a relevant epidemiological role in the link of the sylvatic and domestic *T. cruzi* transmission cycles. Recently, *T. cruzi* Z2 were also recorded from naturally infected marsupials *Philander opossum* and *D. marsupialis* in the State of Rio de Janeiro (Pinho et al. 1994) and from *D. albiventris* from the Chaco forest in Argentina (Wisnisvesky-Colli et al. 1992).

In addition to isoenzyme, RAPD profile was also used to characterize *T. cruzi* from Rio Grande do Sul. The RAPD profiles generated with 4 random primers resulted in complex band patterns, and showed a high genetic heterogeneity of the *T. cruzi* populations studied. A previous extensive analysis of *T. cruzi* strains belonging to distinct zymodemes showed a close correlation between the major groups defined by RAPD and zymodemes (Steindel et al. 1993, Tibayrenc et al. 1993). We obtained similar results when *T. cruzi* Z1, Z2 and ZB strains were compared using primer 3303. In the present study a comparison of the RAPD profiles of 2 Z1 strains generated with 2 primers showed that 74% of the bands were shared. A more extensive analysis of *T. cruzi* Z1 from a wide geographical distribution showed that an average of 55% of the RAPD bands were shared between the strains (Steindel et al. 1993). Using a single primer and a more limited number of strains the authors found that the percentage of bands shared within zymodeme was 73.6% for Z2 and 70.1% for ZB. In the present study, where a more significant number of strains from zymodemes Z2 and ZB, were compared using primers 3307, λgt11R and λgt11F a higher intrazymodeme genetic heterogeneity was evidenced. Using primer λgt11F the percentage of RAPD bands shared within 13 ZB and 13 Z2 strains was respectively 43.3% and 33.3%. However, when 3 primers were used, the bands shared decreased to 20% for ZB and 14% for Z2. The heterogeneity found in our *T. cruzi* ZB and Z2 strains from chronic patients shows that these parasite populations are more polymorphic and demonstrates the analytical power of RAPD. It remains to be observed if there is a relationship between the high genetic heterogeneity of *T. cruzi* strains and different clinical forms of human Chagas' disease in Rio Grande do Sul.

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