The complexity of the sylvatic cycle of *Trypanosoma cruzi* in Rio de Janeiro state (Brazil) revealed by the non-transcribed spacer of the mini-exon gene

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SUMMARY

American trypanosamiasis occurs in nature as a sylvatic cycle, where *Trypanosoma cruzi* interacts with wild triatomines and mammalian reservoirs, such as marsupials, rodents, armadillos and other animals. Due to difficulties in trying to isolate *T. cruzi* stocks from the sylvatic cycle, very few studies have been performed in order to understand the parasite infection in natural environments. Traditionally *T. cruzi* has been considered to be composed of a highly heterogeneous population of parasites. In contrast, the mini-exon and the 24Sα rRNA gene loci have shown that *T. cruzi* stocks can be clustered in 2 major phylogenetic groups: lineage 1 and lineage 2. In this report, 68 recently isolated *T. cruzi* samples from the sylvatic cycle belonging to different geographical areas in Rio de Janeiro, Brazil, have been typed based on a variable spot in the non-transcribed spacer of the mini-exon gene. Eight isolates were from triatomines, 26 stocks were from golden-lion tamarins, 31 from opossums, 2 from rodents and 1 from a three-toed sloth. Thirty (44% – 30/68) isolates were typed as lineage 1, while 36 (53% – 36/68) isolates were typed as lineage 2. Two opossums presented mixed infection. Therefore, 3% (2/68) of the isolates were typed as lineage 1+lineage 2. Using these geographical regions as models of sylvatic environments, it was observed that 96% of the *Didelphis marsupialis* were infected by lineage 2 isolates, while all 26 golden-lion tamarins were infected by lineage 1. The results show preferential association of the 2 lineages of *T. cruzi* with different hosts, composing the complexity of the sylvatic cycle.

Key words: *Trypanosoma cruzi*, mini-exon gene, sylvatic cycle, *Didelphis marsupialis*, *Leontophitecus rosalia*.

INTRODUCTION

Vector-borne transmission of *Trypanosoma cruzi* in humans has been attributed traditionally to 2 connected cycles that have been defined: the sylvatic and the domestic cycles. Human infection via the domestic cycle is a result of the domiciliation of vectors that bring the parasite from the sylvatic environment to human dwellings. The occasional presence of infected sylvatic mammals and bugs in human habitats is also a source for parasite transmission. Furthermore, human infection can be acquired via the sylvatic cycle when humans encroach upon the forest environment (Zeledon *et al*. 1970; Barreto, 1979). Miles *et al*. (1977, 1978, 1980) described 3 distinct zymodemes of *T. cruzi* that correlated with the transmission cycles, Z1 and Z3 with the sylvatic and Z2 with the domestic.

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Intriguing aspects of *T. cruzi* biology are the population heterogeneity and the variability in clinical and epidemiological features of Chagas disease. Molecular and biochemical data generated by several different techniques, such as schizodeme analysis (Morel & Simpson, 1980), multilocus enzyme electrophoresis (Tibayrenc *et al*. 1986), random amplified polymorphic DNA analysis (Tibayrenc *et al*. 1993) and pulsed-field gel karyotyping (Henriksson, Pettersson & Solari, 1993) showed that the *T. cruzi* taxon is composed of heterogenous subpopulations. No correlation has been established between the distinct genotypes or phenotypes and the different aspects of *T. cruzi* and American trypanosomiasis.

Recently, the characterization of 2 different loci (the non-transcribed spacer of the mini-exon gene and 24Sα rRNA gene) of several *T. cruzi* isolates derived from humans, vectors and sylvatic mammals has clustered the stocks into 2 genotypic groups (Souto *et al*. 1996). Further RAPD analysis, studying approximately 60 different anonymous loci, dem-

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Fig. 1. Map of Rio de Janeiro State showing the 5 studied geographical regions – Teresopolis, Jaguanum Island, Miguel Pereira, Itaguai and the Biological Reserve of Poço das Antas (Silva Jardim).

onstrated that these clusters correspond to 2 major phylogenetic divisions (lineage 1 and lineage 2) of the parasite (Souto *et al*. 1996). Molecular epidemiological studies performed in 4 distinct geographical areas in Brazil, analysing 86 field recently-isolated stocks from humans (68) and triatomines (18), showed a preferential association of lineage 1 with the domestic cycle and lineage 2 with the sylvatic cycle (Fernandes *et al*. 1998). These results are consistent with the isozymic dichotomy (Z2 and Z1/Z3) proposed by Miles *et al*. (1978, 1980). However, a model in which distinct genotypes (lineage 1 or lineage 2) are restricted to either the domestic or the sylvatic cycle does not explain how these cycles are connected. Recently, experimental data derived from 157 *T. cruzi* isolates collected in non-endemic and endemic regions of Chagas disease in Brazil allowed the proposition of a hypothesis that could clarify the linkage between the domestic and the sylvatic cycles (Zingales *et al*. 1998).

To re-evaluate the sylvatic cycle of *T. cruzi*, in the light of the recent division of this protozoan into 2 major lineages, we have typed 68 isolates, collected recently from sylvatic mammals and wild bugs, from different geographical areas in Rio de Janeiro State, a Brazilian region with no cases of autochthonous Chagas disease.

MATERIALS AND METHODS

Mammal capture, vector collection, parasite isolation and culture

Animals from 5 areas of the Atlantic Coast Rainforest of Rio de Janeiro State: Silva Jardim (Poço das Antas Biological Reserve), Teresopolis, Jaguanum Island, Miguel Pereira and Itaguai (Fig. 1), were captured in baited traps. After appropriate anaesthesia with 13 mg/kg body weight kethamine acetate, blood was collected by venopuncture and cultured in duplicate tubes containing NNN-medium supplemented with a LIT medium (Camargo, 1964) overlay. The animals captured in the Biological Reserve were released to the environment after the blood collection. On the other hand, all the animals that were captured in the other regions were studied in Fiocruz Animal Facilities (Ministry of Health, Brazil). The mammals that were positive for *T. cruzi* infection were prospectively followed up, searching for eventual clinical manifestations. The negative animals were released to the original geographical area.

The parasite cultures were examined fortnightly over a period of 4 months. Parasites from positive cultures were amplified in LIT medium. Approximately 2 sets of 100 μl of growing culture (10^9 parasites/ml) were centrifuged at 4000 ***g*** and the cells were washed 3 times with PBS buffer. The final pellets were stored in liquid nitrogen until used.

Triatomines were collected manually and found exclusively in Silva Jardim and Teresopolis. Parasites from positive faeces were inoculated intraperitoneally in outbred Swiss male mice in order to isolate the parasite and were recovered from the mice by haemoculture and *in vitro* amplification as described above.

The parasites isolated from mammals and vectors were characterized as *T. cruzi* due to morphological features and also by the parasitemic curves in the infected mice.

DNA extraction and mini-exon genotyping

Genomic DNA was extracted from cultured cells (Pharmacia DNA extraction kit) following the manufacturer's instructions. A portion of the non-transcribed spacer of the mini-exon gene was amplified by the polymerase chain reaction using the oligonucleotides and reaction conditions described elsewhere (Souto *et al*. 1996). The amplification products were analysed by electrophoresis through 2% agarose gel, followed by ethidium bromide staining and UV visualization.

RESULTS

To examine the distribution of the 2 lineages of *T. cruzi* in the sylvatic cycle, a total of 394 animals from 5 different mammalian orders (Marsupialia – 130 *Didelphis marsupialis*, 42 *Philander opossum*, 17 *Marmosa cinerea*, 4 *Metachyrus nudicaudatus* and 1 *Caluromys philander*, Primata – 172 *Leontopithecus rosalia* (golden lion tamarin); Rodentia – 7 *Nectomys squamipes*, 6 *Holochilus brasiliensis*, 2 *Coendou villosus* and 4 non-identified rodents; Chiroptera – 6 non-identified bats and Edentata – 2 *Bradipus torquatus* and 1 *Dasypus novemcinctus*) were captured in 5 distinct localities of Rio de Janeiro State (Fig. 1). Only marsupials were captured in Teresopolis,

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Table 1. *Trypanosoma cruzi* infection in marsupial species captured in different localities of Rio de Janeiro State

(The rate of infection is given by: number of *T. cruzi* positive marsupials/total number of animals.)

	Teresopolis	Jaguanum Island	Miguel Pereira	Itaguai	Silva Jardim	Total
Didelphis marsupialis	21/35 (60%)	34/38 (90%)	4/14 (29%)	3/28 (11%)	2/15 (13%)	64/130 (49%)
Philander opossum	18/36 (50%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/6 (0%)	18/42 (43%)
Marmosa cinerea	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/17 (0%)	0/17 (0%)
Metachyrus nudicaudatus	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/4 (0%)	0/4 (0%)
Caluromys philander	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/1 (0%)	0/1 (0%)
Total	39/71 (55%)	34/38 (90%)	4/14 (29%)	3/28 (11%)	2/43 (5%)	82/194 (42%)

Table 2. Genotyping of *Trypanosoma cruzi* isolates from mammals and vectors captured in 5 different geographical areas of Rio de Janeiro

Mammals	Lineage 1	Lineage 2	Lineage 1+2	Total
Didelphis marsupialis	0 (0%)	22 (96%)	2 (4%)	24
Philander opossum	3 (43%)	4 (57%)	0 (0%)	7
Leontopithecus rosalia	26 (100%)	0 (0%)	0 (0%)	26
Holochilus brasiliensis	0 (0%)	2 (100%)	0 (0%)	2
Bradipus torquatus	0 (0%)	1 (100%)	0 (0%)	1
Vectors				
Rhodnius sp.	0 (0%)	7 (100%)	0 (0%)	7
Triatoma vitticeps	1 (100%)	0 (0%)	0 (0%)	1
Total	30	36	2	68

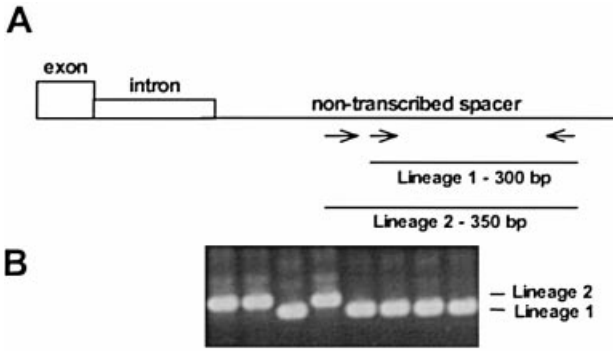

Fig. 2. (A) Scheme of the mini-exon gene and the amplification of the variable spot in the non-transcribed spacer. The arrows correspond to the primers used in the PCR generating a 300 bp or a 350 bp product when DNA samples from *Trypanosoma cruzi* lineage 1 or 2 were typed, respectively. (B) Agarose gel (2%) electrophoresis of the amplified products stained by ethidium bromide and visualized under UV light.

Jaguanum Island, Miguel Pereira and Itaguai. In Silva Jardim, animals from all the orders were captured.

Blood samples were taken and cultured for trypanosomes. The haemocultures were positive for protozoa morphologically similar to *T. cruzi* in 121 mammals. Among the captured marsupials, the rates of infection detected by the haemoculture procedure (Table 1) were 55% (39/71) in Teresopolis, 90% (34/38) in Jaguanum Island, 29% (4/14) in Miguel Pereira, 11% (3/28) in Itaguai and 5% (2/43) in Silva Jardim. In this latter district, 20% (35/172) of the primates, 16% (3/19) of the rodents and 50% (1/2) of the Edentata were also positive. No evidence of infection by *T. cruzi* was found among the 6 bats.

T. cruzi was also isolated from 11/23 *Rhodnius* sp. found in Teresopolis and from the only *Triatoma vitticeps* collected in Silva Jardim.

Of the 133 mammalian and insect isolates, 68 were further characterized into the 2 lineages of the basis of DNA sequence variability in the non-transcribed spacer of the mini-exon gene (Fig. 2 and Table 2). This nuclear gene is a multicopy tandem repeat sequence, involved in the trans-splicing phenomenon where the 39 nucleotides of the canonical exon are added to the 5′-end of all mRNAs (Parsons *et al.* 1984). The non-transcribed spacer of this gene in *T. cruzi* is composed of 500 bp of which only 30 bp are required for promoter activity (Nunes *et al.* 1997). Therefore, the other 470 bp have no identified function and, while retaining the same length among many isolates, have accumulated multiple base changes. Indeed, the hypervariability in the non-transcribed spacer of the mini-exon gene has proven to be a useful molecular marker to define different kinetoplastic species (Fernandes, Degrave & Campbell, 1993; Fernandes *et al.* 1994; Sturm, Fernandes & Campbell, 1995). Of the 68 isolates

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tested, 44% (30/68) were typed as lineage 1 and 53% (36/68) were typed as lineage 2. Two of the isolates from *D. marsupialis* were typed as lineage 1+lineage 2(3%; 2/68). This result is interpreted as representing mixed infections.

In Silva Jardim, all 26 golden lion tamarins and the triatomine (*T. vitticeps*) possessed *T. cruzi* typed as lineage 1, while all the other animals (2 *D. marsupialis*; 2. *H. brasiliensis*; 1 *N. squamipes* and 1 *B. torquatus*) yielded *T. cruzi* isolates from lineage 2. In Teresopolis, the 2 *D. marsupialis* showed mixed infections and parasites belonging to lineage 1 were observed in 3 *P. opossum*. All the other mammals and vectors (7 *D. marsupialis* and 4 *P. opossum* and 7 *Rhodnius sp.*) were infected by parasites of lineage 2. Opossums of the genus *Didelphis* from the different geographical origins were all infected by parasites of lineage 2 (Fig. 2 and Table 2).

DISCUSSION

The Atlantic Coast Rainforest is an ideal ecotope to study the *T. cruzi* sylvatic transmission cycle due to its richness in mammalian fauna and the protection performed by governmental organization (IBAMA) that controls hunting and human colonization.

Marsupials are classically considered as the most important and ancient sylvan reservoir of *T. cruzi* and may represent the linkage between both the domestic and sylvatic cycles as it adapts to the human dwellings. A significant difference among the rates of infection in the marsupials within the distinct areas was observed. For example, in Jaguanum Island, 90% of the marsupials (34/38) were naturally infected in contrast to only 5% (2/43) in Silva Jardim. In this latter district, the primates comprise the most important reservoir group as they showed to be 20% (35/172) infected. These data indicate that concerning the transmission of *T. cruzi* and most probably other zoonosis, each ecotope should be considered as a unique epidemiological unit and any theoretical generalization may lead to misinterpretation.

In the *T. cruzi* taxon, the mini-exon gene, in association with the 24Sα rDNA and RAPD analysis, has discriminated 2 major genetic lineages (Souto *et al*. 1996). Previous epidemiological studies using these markers showed a preferential association of lineage 1 with the domestic transmission cycle and lineage 2 with the sylvatic one (Souto *et al*. 1996; Fernandes *et al*. 1998; Zingales *et al*. 1998). This definition is in agreement with former conclusions indicating the association of particular zymodemes with the 2 transmission cycles (Miles *et al*. 1978, 1980). However, the present study reveals that the sylvatic cycle is more complex than previously assumed since both *T. cruzi* lineages are encountered in similar ecotopes belonging to the Atlantic Coast Rainforest in Rio de Janeiro (Miles *et al*. 1980; Pinho *et al*. 1996, 1997). Concerning marsupials, all *D. marsupialis* (22 samples) presented lineage 2 parasites except for 2 animals from Teresopolis that showed mixed infections (lineage 1+lineage 2). In this same region 4 out of 7 *P. opossum*, another marsupial species, harboured parasites of lineage 2 while the remaining 3 contained *T. cruzi* lineage 1. In Silva Jardim, all the mammals except the primates were shown to be infected by parasites of lineage 2. Interestingly all the 26 golden lion tamarins presented exclusively parasites typed as lineage 1.

Triatomine vectors were captured in Silva Jardim (1 specimen) and Teresopolis (7 insects). In this latter case all specimens were classified as *Rhodnius* sp. and found to be infected with lineage 2 parasites. This observation suggests that this vector is involved in the transmission cycle among the lineage 2 infected mammals in Teresopolis. In Silva Jardim, at least the only captured *T. vitticeps* is probably involved with the transmission cycle of the primates.

Clark & Pung (1994), studying polymorphisms in the small subunit of the rRNA gene, described that in southern Georgia (USA) 13 *T. cruzi* stocks isolated from raccoons (*Procyon lotor*) showed the same riboprint while 4 *D. marsupialis* were infected by parasites of a different genetic pattern. The riboprint presented by the parasites infecting the raccoons corresponds to lineage 1 and the protozoa isolated from opossums correspond to lineage 2. Further studies performed in St Catherine's Island (Georgia, USA), typing 7 *T. cruzi* isolates from lion-tailed macaques (*Macaca silenus*) and 1 stock isolated from a ring-tailed lemur (*Lemur catta*) showed that these parasites display the same riboprint as those isolated from raccoons, and consequently lineage 1 (Pung *et al*. 1998).

In accordance with the analysis of Pung and co-workers (Clark & Pung, 1994; Pung *et al*. 1998) regarding sylvatic regions in Georgia (USA), our data concerning the Atlantic Coast Forest in the State of Rio de Janeiro (Brazil) indicate the presence of both *T. cruzi* lineages infecting wild mammals of the same ecotope. Further, a preferential linkage of a specific parasite lineage to a particular mammalian order was observed. In our case, *D. marsupialis* was preferentially infected by *T. cruzi* lineage 2 while primates were infected by lineage 1 parasites. These data could be explained by the possibility that animal species act as biological filters selecting a given parasite genotype and by the development of 2 distinct cycles of *T. cruzi* performed by each of the lineages in those regions (Deane *et al*. 1984*a*, *b*; Dvorak *et al*. 1988; Macedo & Pena, 1998). The finding that lineage 1 is well adapted to primates may explain why this lineage is prevalent in human infection, at least in Brazil. In fact, we have recently shown that *T. cruzi* isolated from humans in traditional areas for Chagas disease, such as Minas Gerais, Paraiba and Piaui are preferentially lineage

1, showing a statistically significant association of this genotype to the domestic cycle (Fernandes *et al*. 1998). Probably the linkage between the 2 cycles (sylvatic and domestic) in these areas is only possible due to the maintenance of lineage 1 in nature by reservoirs, such as the primates, *P. opossum* and raccoons. Although there is no autochthonous Chagas disease in Rio de Janeiro State, the presence of triatomines that easily adapt to human dwellings, and primates and opposums infected with lineage 1, are the characteristics that compose the scenario for the establishment of a domestic cycle and therefore the appearance of human infection.

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