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Molecular and Proteolytic Profiles of *Trypanosoma cruzi* Sylvatic Isolates from Rio de Janeiro-Brazil

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1. Introduction

Chagas disease, also known as American trypanosomiasis, has its epidemiology conditioned to the (i) triatominae vectors, (ii) etiologic agent, *Trypanosoma cruzi*, and (iii) sylvatic and sinantropic reservoirs, the mammals. Social factors associated with economic factors, such as industry development, population growth and rural area colonization, which lead directly to ecological imbalance, provide favorable conditions for the disease establishment (Barretto, 1967; Ávila-Pires, 1976).

In 1909, Carlos Chagas releases his discovery on a new human disease, the American trypanosomiasis, subsequently known as Chagas disease. Carlos Chagas described the etiologic agent, the protozoan belonging to the Trypanosomatidae family *Trypanosoma cruzi*, and its insect vector belonging to the Hemiptera order, Triatominae subfamily, the so-called kissing bug (Chagas, 1909; Lent & Wygodzinsky, 1979).

The natural history of the Chagas disease probably started millions of years ago probably as a sylvatic enzooty, and it is still present in different areas from Brazilian territory. The arrival of men in these areas, as well as comprehensive deforestation caused by extensive farming during the past 300 years has caused triatomine insects, formerly sylvatic animal blood-sucking bugs, to meet men (Ferreira et al., 1996; Coura, 2007). Hence, the disease was characterized as a zoonosis, when men invaded the sylvatic habitat, deforesting and changing the ecological balance, and making triatomine bugs access to the residences.

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Therefore, the transmission cycle of *T. cruzi* is comprised by a sylvatic cycle, in which the parasite circulates among mammals and sylvatic vectors, and a domiciliary cycle, in which the infection is ensued by the contact of mammals, sylvatic vectors and sinantropic animals with domestic and domiciled animals, including men (Barretto, 1979).

Human Chagas disease, an antropozoonosis that evolved from a zoonosis, is strongly related with men's social class, type of work and habitation (Dias, 2000). During the 70's, the disease endemic area achieved at least 2,450 Brazilian cities, 771 of which were detected to have *Triatoma infestans*, the main disease vector in Brazil. At that time, there were over five million people affected by the disease in the country, with an incidence of approximately one hundred thousand new cases yearly and mortality above ten thousand deaths yearly. Less than five percent of blood banks used to control donors and over seven hundred cities had their homes infected by *T. infestans*. This situation led scientists to press the government to prioritize a national program against the disease. Homes from endemic areas were sprinkled with the appropriate insecticide and, in accordance with law; mandatory screening of blood donors was implemented throughout the country (Dias et al., 2002). The control program of the main vector in Brazil was recognized in 2006, with a certificate from the World Health Organization (WHO) for virtual elimination of *T. infestans* in Brazil (Dias, 2006). As the main vector was eliminated, currently there is a concern that other Triatominae species, formerly deemed secondary in the disease transmission, such as *Triatoma brasiliensis*, *Triatoma pseudomaculata* and *Panstrongylus megistus*, take the place of *T. infestans* in some locations, therefore becoming potential disease vectors in Brazil (Coura, 2009).

Despite the great progress in controlling vector and transfusion transmission in the countries from the Southern Cone, transmission is ongoing in other parts of the continent, and the issue of already infected people, most of whom are in the chronic phase of the disease, is still a challenge to public health (Urbina, 1999). Currently Chagas disease affects between twelve and fourteen million people in Latin America, and at least 60 million people live in areas with transmission risk (WHO, 2002). In Brazil, the disease notification became compulsory as per Ordinance V of Health Surveillance Secretary of Ministry of Health dated February 21, 2006.

2. Triatomines

The first report of triatomine existence was recorded by the Spanish Francisco López de Gomara, in 1514, when mentioning Darién region he said: "Hay muchas garrapatas y chinches com alas", apparently referring to *Rhodnius prolixus* (Stål, 1859) (León, 1962). *Cimex rubrofasciatus* (*Triatoma rubrofasciata*), was described in 1773 by De Geer, and later assigned by Laporte as the type species of *Triatoma* genus (Lent & Wygodzinsky, 1979). In Brazil, the first report of triatomine in domicile was possibly *Panstrongylus megistus* (Burmeister, 1835) (Gardner, 1942). However, the identification of *Trypanosoma cruzi* sylvatic isolates is contemporary to the discovery of this parasite and Chagas disease by Carlos Chagas in 1909. When they went to Lassance, Minas Gerais, Brazil, for malaria epidemics study, he identified flagellated forms in the intestine of triatomine of *Conorhinus megistus* (*Panstrongylus megistus*) in humans and cats, referring to them as *Schizotrypanum cruzi* (Chagas, 1909). Later Chagas (1912) isolated the parasite in armadillos (*Tatusia novemcincta*, now called *Dasypus novemcinctus*), identifying the *T. cruzi* sylvatic reservoirs, and in the

same ecotope he found infected *Triatoma geniculata* (*Panstrongylus geniculatus*) specimens, establishing the disease sylvatic cycle (Coura & Dias, 2009).

Between 1913 and 1924 it became evident that the disease was not restricted to Brazil, being diagnosed in other countries in Central and South Americas, such as El Salvador, Venezuela, Peru and Argentina (Talice et al., 1940; Zeledón, 1981). In subsequent studies, Coura & Dias, 2009 mentions that Chagas (1924) demonstrated *T. cruzi* transmission cycle in the Amazon region with the identification of this parasite in monkeys of *Saimiri scirius* species.

In Rio de Janeiro state, the first Triatominae occurrence dated 1859, when Stal described *Conorhinus vitticeps* species, now called *Triatoma vitticeps*. At that time, Rio de Janeiro was assigned as type location, without defining whether it referred to the city or state.

Following this finding, Neiva (1914) recorded the occurrence of *T. vitticeps* in Conceição de Macabu, formerly Macaé city district, presently Conceição de Macabu city. Due to information accuracy, Lent (1942) suggested it would be considered as the type location of *T. vitticeps*.

Subsequently, Pinto (1931, as cited in Lent, 1942) pointed out its presence in Magé, and Lent (1942) in Nova Friburgo, at Secretario location in Petrópolis city and at Federal District, which was Rio de Janeiro at that time. In Minas Gerais state, it was observed by the first time by Martins et al (1940), and in Espírito Santo state, as mentioned by Lent (1942).

In Rio de Janeiro state other species were also found. Guimarães and Jansen (1943) collected *Panstrongylus megistus* specimens in a building by the hill, and identified *Trypanosoma cruzi* sylvatic reservoir (skunk), but did not find the sylvatic focus. Dias (1943) listed Chagas disease transmitters in Rio de Janeiro as being *Panstrongylus megistus*, *Panstrongylus geniculatus* (Latreille, 1811), *Triatoma vitticeps* (Stal, 1859), *Triatoma oswaldoi* (Neiva & Pinto, 1923), *Triatoma infestans* (Klug) and *Triatoma rubrofasciata* (De Geer, 1773), first recording the occurrence of *Schizotrypanum sp*-infected *P. megistus* in two districts in the capital of Republic (Santa Tereza and Botafogo). In 1953, in a survey performed at Araruama and Magé, Dias stated it was a relevant issue for the State, while Bustamante & Gusmão 1953 pointed out the presence of *T. infestans* at Resende and Itaverá cities. New findings have been identified, such as that of Coura et al. (1966), who found *P. megistus*, *Triatoma tibiamaculata* and *T. rubrofasciata* in three districts at Rio de Janeiro city, and that of Aragão & Souza (1971), who signalized the presence of *T. infestans* colonizing domiciles at two cities in Baixada Fluminense. In the same year, Coura et al. (1966) described some autochthonous instances of *T. infestans*-transmitted Chagas disease at Baixada Fluminense, and Becerra-Fuentes et al. (1971) recorded *T. rubrofasciata* occurrence at Morro do Telégrafo in the former Guanabara state. Silveira et al. (1982) performed an entomologic inquiry at Duque de Caxias and Nova Iguaçu cities (RJ), and only found *T. infestans* species. Ferreira et al. (1986) verified the occurrence of *T. vitticeps*, and positivity for *T. cruzi*-like forms, in 12 cities, of which the one with the highest incidence for both observations was Triunfo location at Santa Maria Madalena city. In 1989, a *P. geniculatus* specimen was found in a domicile at São Sebastião do Alto city (RJ) (personal communication with Teresa Cristina M. Gonçalves). The occurrence of *Rhodnius prolixus* (Stål, 1859) in Teresópolis was pointed out by Pinho et al. (1998), which caused questioning, once this species was restricted to the northern region of the country. Nowadays it is known this species does not occur in Brazil (Monteiro et al., 2000, 2003). *T. vitticeps* was found in Poço das Antas, Silva Jardim city, by Lisbôa et al. (1996), and in Santa Maria Madalena by Gonçalves et al. (1998). In both

locations, biological and morphological characterization of *T. cruzi* isolates, obtained for both triatomine bugs and vertebrate hosts, confirmed the maintenance of enzootic disease form. In the period from 2008 to 2010 *T. vitticeps* was pointed out at Cantagalo, Tanguá, Trajano de Morais, and São Fidélis cities (Oliveira et al., 2010).

In Espírito Santo, where *T. vitticeps* incidence was also signaled, the rates of infection by *T. cruzi*-like forms were assessed in specimens collected in the domicile: 4% by Santos et al. (1969) at Alfredo Chaves (ES); 25.2% by Silveira et al. (1983) at Cachoeiro do Itapemirim and Guarapari (ES); 35.2% by Ferreira et al. (1986) in 12 cities from Rio de Janeiro state; 64.70% by Sessa & Carias (1986) in 19 cities from Espírito Santo state; and 70.2% and 51.8%, respectively, for females and males, by Dias et al. (1989).

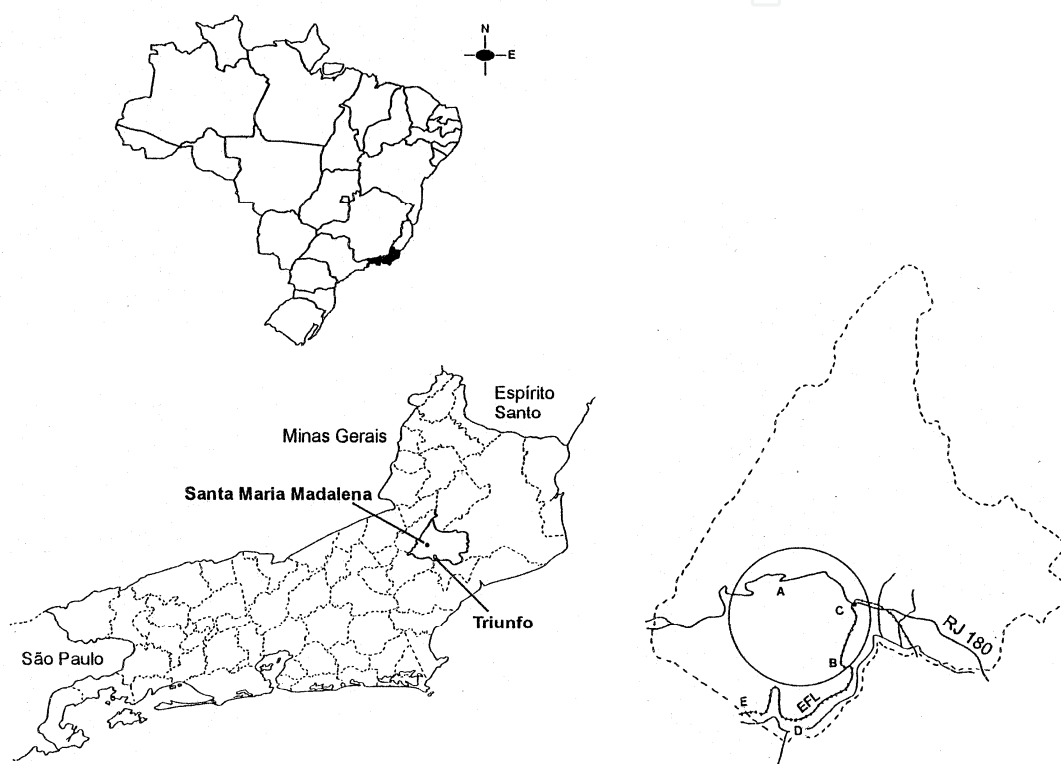


Fig. 1. Studied area and sites of capture of *Triatoma vitticeps* in Triunfo, Santa Maria Madalena, Municipal district, State of Rio de Janeiro, Brazil.

Data from National Health Foundation (“FUNASA”) signaled *T. vitticeps* presence in the northern region of Rio de Janeiro state, and the number of notifications on adult form occurrence was increasing (Lopes et al., 2009; Dias et al., 2010). Although studies regarding *T. vitticeps* biology have suggested that this species would not represent a major concern from epidemiologic point of view (Dias, 1956; Heitzmann-Fontenelle, 1980; Silva, 1985; Diotaiuti et al., 1987; Gonçalves et al., 1988, 1989), reports of this species frequently invading the domicile with high *T. cruzi* infection rates (Gonçalves et al., 1998, Gonçalves, 2000) indicated its study was required. With sylvatic habit and unknown habitat, this species ecobiology was studied in further details at Triunfo district, Santa Maria Madalena city (RJ), in three areas (A, B and C) (Figure 1). Of the triatomine bugs collected, 68 *T. cruzi* samples

were isolated, which showed heterogeneity in which refers to biology, histopathogenesis and differential expression of surface enzymes.

2.1 *Trypanosoma cruzi*

Trypanosoma cruzi (Figure 2) is a flagellated protozoan belonging to Trypanosomatidae family (Kent, 1880), Kinetoplastida order, *Trypanosoma* genus (Chagas, 1909a; Coura, 2006). Kinetoplastida order was established as a function of the presence of a single cytoplasmic structure, the kinetoplast (Wallace, 1966), where mitochondrial DNA or k-DNA is concentrated. Its form, size, and position are important for characterizing the different evolution forms of the parasite (Vickerman, 1985).

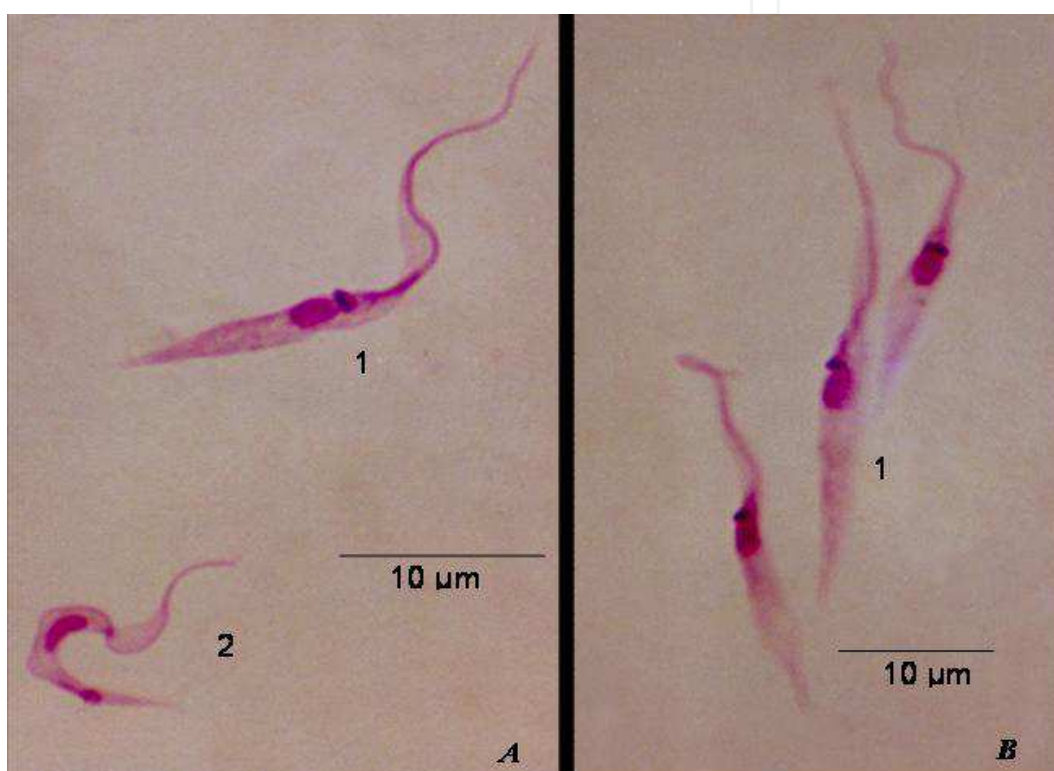


Fig. 2. Epimastigote (1) and tripomastigote (2) forms of *Trypanosoma cruzi* sylvatic isolates from Trinfo, Santa Maria Madalena municipal district, State of Rio de Janeiro - Brazil.

It is a euryxene and digenetic trypanosomatid, since part of its life cycle occurs inside a vertebrate or invertebrate host (Hoare, 1964). Vertebrate and invertebrate hosts are represented, respectively, by domiciled or domestic mammals and sylvatic triatomines.

The parasite cycle can be summarized as follows: the triatomine vector usually defecates during or at the end of blood sucking, eliminating metacyclic trypomastigote forms of *T. cruzi* on the vertebrate hosts. These forms found in dejections can penetrate the host through a continuity skin solution or skin mucosa. Inside the host cell, trypomastigotes transform into amastigotes and, approximately 35 hours later, the binary division begins. After five days, amastigotes transform into trypomastigotes, and as soon as they have long flagella, the cell disrupts releasing these forms into the bloodstream, so that they infect other cells or achieve different organs (Sousa, 2000). In triatomines, the blood-sucking trypomastigote

forms ingested during hematophagy differentiate into epimastigotes in the digestive tract. Another differentiation occurs in the digestive tract, more specifically in its final portion and in rectus, when epimastigotes transform into metacyclic trypomastigotes, which is infectious for the vertebrate host and eliminated with the feces (Zeledón et al., 1977; Garcia & Azambuja, 2000).

T. cruzi is found as a parasite in a considerable number of mammals and in a wide range of tissues and niches in these hosts (Deane et al., 1984). Such eclecticism has characterized *T. cruzi* as one of the most successful microorganism in presenting parasitary life (Jansen et al., 1999). Therefore, this protozoan comprises a wide set of heterogeneous populations that circulate through very diverse vertebrate and invertebrate hosts, with a variation of different genotype predominance. The parasite has several morphological, physiological and ecological variations, and also in which refers to its infectivity and pathogenicity (Miles et al., 1978, 1980, 2009), which can warrant the various clinical manifestation forms of Chagas disease observed in different geographic regions (Miles et al., 1981a). Many studies have been performed seeking molecular markers that could correlate the parasite genotype with varying types of this infirmity clinical manifestation. Several works tried to clarify the multiple factors related with population epidemiology and genetics.

T. cruzi has a great phenotypic and genotypic variability in its strains, and therefore this protozoan has the ability to perform genetic exchanges through an unusual mechanism of nuclear fusion, forming a polyploidy progeny, which can suffer recombination among alleles, and after losing its chromosome, can return to diploid status. Some studies provided strong evidence that sexual reproduction is absent in *T. cruzi*, and that its population structure is clonal (Gaunt et al., 2003; Lewis et al., 2009).

3. Molecular profile of *T. cruzi* populations

Early investigations on the genetic of *T. cruzi* populations are based on electrophoretic profiling of isoenzymes (zymodeme analysis), a technique used to explore the genetic diversity of microorganisms. Enzymatic electrophoresis uses soluble raw-materials and extracts from an organism to assess the activity of a protein, and its product is revealed by means of a colorimetric reaction. Under controlled conditions, differences in isoenzymatic mobility imply genetic differences (Miles, 1985; Miles & Cibulkis, 1986). Toyé (1974) was the first to use isoenzymes to classify trypanosomas from the New World, reporting differences among *T. cruzi* samples. By the end of the 70's and beginning of the 80's, several studies on isoenzymatic variability among *T. cruzi* populations were performed in Brazilian Northeast, and later in different regions within the country, by employing six enzymes: ALT (alanine aminotransferase), AST (aspartate aminotransferase), glucose phosphate isomerase (GPI), glucose-6-dehydrogenase phosphate (G6PDH), malic enzyme (ME) and phosphoglucumutase (PGM), characterizing three enzymatic profiles belonging to parasite groups called zymodemes I (Z1), II (Z2) and III (Z3). Z1 and Z3 are related with the sylvatic transmission cycle and Z2 with the domestic transmission cycle of the parasite (Miles et al., 1977, 1978, 1980, 1981a, b). As the number of analyzed isoenzymes has been amplified and sub-populations circulating among domestic and sylvatic vertebrates and invertebrates have been studied, an elevated degree of *T. cruzi* heterogeneity was verified (Miles et al., 1980; Bogliolo et al., 1986; Tibayrenc et al., 1986; Tibayrenc & Ayala, 1988; Barnabe et al., 2000).

With technologic advancement and the discovery of new molecular biology tools, it was possible to study the diversity of *T. cruzi* by means of DNA analysis, allowing for molecular characterization of this parasite strains (Devera et al., 2003). Therefore, the genetic diversity was corroborated by randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analyses, DNA fingerprinting, microsatellites and molecular karyotyping (reviewed by Zingales et al., 1999). Analyses of gene sequences with lowest evaluative rates, such as ribosomal RNA genes, classic evolution markers and mini-exon genes, indicated dimorphism in *T. cruzi* isolates, rating them into two groups (Souto et al., 1996). Mini-exon gene that is present in Kinetoplastid nuclear genome at approximately 200 copies in a tandem type array is composed by three different regions: exon, intron and intergenic regions. Exon is a highly preserved sequence between de order compounds, added to nuclear messenger RNA post-transcription (Devera et al., 2003). Intron is moderately preserved between species of the same genus or sub-genus, and the intergenic region is particularly different among species. In *T. cruzi*, the amplification of mini-exon intergenic region by Polimerase Chain Reaction (PCR) allowed us to classify the different isolates into two main taxonomic groups: *T. cruzi* I and *T. cruzi* II (Fernandes, 1996; Souto et al., 1996; Fernandes et al., 1998). Thereafter, PCR amplification assay were standardized, allowing for rapid molecular typing, which started to be broadly used. Thereby the use of multiplex PCR based on intergenic region allowed us to classify the isolates as *T. cruzi* I, *T. cruzi* II, *T. cruzi* Z3 or *T. rangeli* with 200, 250, 150 pb and 100 pb, respectively (Fernandes et al., 2001a).

Aiming at standardizing double lines and hybrid isolates, a committee settled the lines were referred to as *T. cruzi* I and *T. cruzi* II "groups" (Zingales et al., 1999). Such denomination was not attributed to hybrid isolates, and additional studies are recommended to better characterize them (Zingales, 2011). From hybrid isolate gene sequence analysis, it has been shown that events of genetic exchanges with these parasites originated four distinct isolate groups (Sturm & Campbell, 2009). Thus, by using multilocus enzyme electrophoresis (MLEE) and RAPD markers, it was suggested that the group *T. cruzi* II was divided into five subgroups, including the four hybrid groups (Freitas et al., 2006; Brisse et al., 2000). *T. cruzi* III, a third ancestral group, was proposed from the analysis of microsatellites and mitochondrial DNA.

In 2009, the scientific community felt the need to standardize once again *T. cruzi* groups' nomenclature, aiming at clarifying questions on biology, eco-epidemiology and pathogenicity (Zingales et al., 2009). In this respect, it was recommended that *T. cruzi* was divided into six groups (*T. cruzi* I–VI), and that each group was called Discreet Taxonomic Units (DTUs) I, IIa, IIb, IIc, IId, IIe (Figure 3), defined as groups of isolates that are genetically similar and can be identified through molecular or immune markers (Tibayrenc, 1998), with DTU I corresponding to *T. cruzi* line I and DTU IIb corresponding to *T. cruzi* line II, and sub-lines IIa and IIc-e associated with hybrid strains and those belonging to zymodeme 3 (Brisse et al., 2000). The distribution of haplotypes from five nuclear genes and one satellite DNA was analyzed in isolates that were representative of the six DTUs by net genealogy and Bayesian phylogeny. Such data indicated that DTUs *T. cruzi* I and *T. cruzi* II are monophyletic and the other DTUs have different combinations of *T. cruzi* I and *T. cruzi* II haplotypes and DTU-specific haplotypes (Tomazi et al., 2009; lenne et al., 2010). One of the possible interpretations for this observation is that *T. cruzi* I and *T. cruzi* II are two different species and that DTUs II-IV are hybrid resulting from independent hybridization/genomic combination events (Zingales, 2011).

In this setting, the characterization of these parasites extracted from different hosts aim at helping clarify the biological meaning and repercussion of this variability for clinics and for Chagas disease epidemiology (Lainson et al., 1979). However, the great majority of studies performed are related to parasite populations belonging to TCI and TCII groups, with scarce works performed with Z3 group.

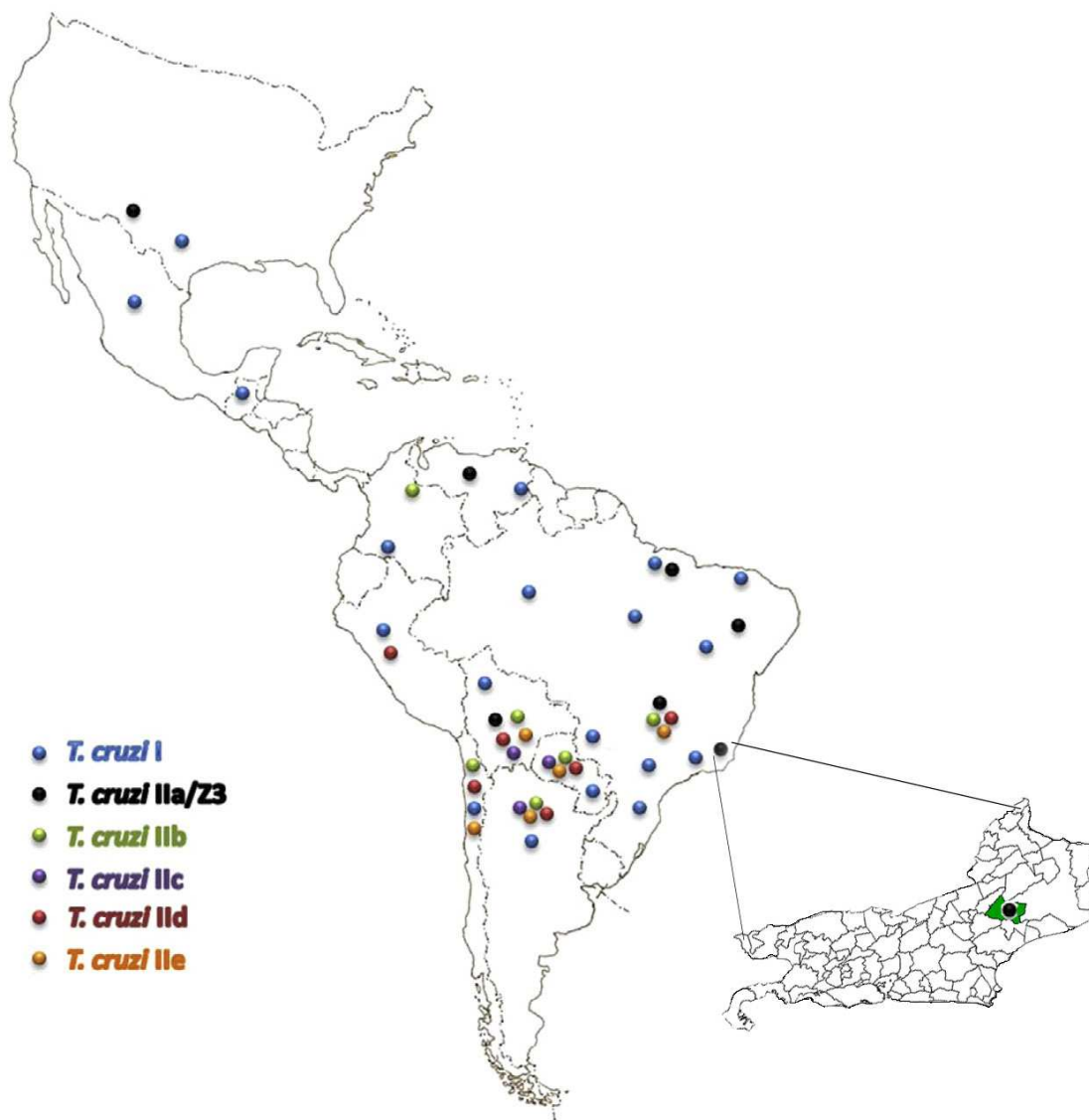


Fig. 3. General pattern of distribution of *T. cruzi* lineages and sublineages; the sylvatic isolates from Rio de Janeiro (extended map showing in green Triunfo, Santa Maria Madalena municipal district) were typed as *T. cruzi* IIa/Z3. (Adaptated map by Noireau F. Vet. Res. (2009)).

3.1 *T. cruzi* isolates from Rio de Janeiro

Therefore, this work was performed from *T. cruzi* samples isolated from *Triatoma vitticeps* (Figure 1) by Gonçalves in 2000, at Triunfo location, 2nd district of Santa Maria Madalena city, Rio de Janeiro state (Figure 2). Four hundred sixty five (465) *Triatoma vitticeps* specimens were collected: 294 females, 156 males, and 15 nymphs from five different areas:

area A, located at 250-meter altitude and 3.5 km distant from the district headquarters, very modified by deforestation for banana farming; area B, located at 130-meter altitude and 4 km distant from the headquarters, placed in a valley with preserved vegetation (secondary forest). These areas are 2-km distant to each other, separated by a mountain (Figure 3). Area C, the district headquarters, at 40-meter distance, was totally modified by pasture formation, and areas D and E were totally preserved and placed at 10 and 12-km distances from the headquarters, respectively. *T. cruzi* isolates used in this study were extracted from triatomines captured from areas A, B and F (Table 1). Area F was located in Vista Alegre, a city neighboring Conceição de Macabu, at Northern region of Rio de Janeiro State (Gonçalves, 2000).

Isolates (Samples)	Area	Host	Geographical origin
SMM10	A	Tv	Triunfo
SMM53	A	Tv	Triunfo
SMM88	A	Tv	Triunfo
SMM98	A	Tv	Triunfo
SMM36	B	Tv	Triunfo
SMM82	B	Tv	Triunfo
SMM1	F	HCD	Conceição de Macabu

SMM (Santa Maria Madalena)

Tv - *Triatoma vitticeps*; HCD (Haemoculture of the swiss mouse) - the parasites were inoculated in mice and was done haemoculture.

Table 1. *Trypanosoma cruzi* samples isolated from *Triatoma vitticeps* captured on the State of Rio de Janeiro, Brazil

Those *T. cruzi* samples isolated from *Triatoma vitticeps*, collected in Rio de Janeiro State, were classified by our group as Z3 based on mini-exon gene (Santos-Mallet et al., 2008) and showed great heterogeneity regarding growth curve and mouse virulence patterns (Silva, 2006), susceptibility to benznidazole (Sousa, 2009), total protein pattern and proteolytic activity profile (Gomes et al., 2006; Gomes et al., 2009). This heterogeneity observed in samples collected from the same region leads to questionings on how this diversity could influence the parasite-host cell interaction.

3.2 Molecular profile of *T. cruzi* isolates from Rio de Janeiro

The results obtained by means of molecular analysis revealed that the isolates have similar profiles, except for sample SMM1 (area F). Samples SMM10, SMM53, SMM88, SMM98 (area A), SMM36 and SMM82 (area B) revealed the presence of 150 bp, indicating that they belong to the zymodeme III group (Z3; Figure 4). Likewise, sample SMM1 from area F showed similarity to Z3 (150 bp), but also presented another band that may be related to the TcII profile (250 bp) and was very similar to the reference strain CL Brener (Figure 4). The phylogenetic position of Z3 has been much debated. According to some authors, the numerical taxonomy based on 24 isoenzymatic Z3 profiles is more closely associated with Z1 (TcII) than with Z2 (TcI) (Ready & Miles, 1980). However, other works place Z3 in an intermediate position between Z1 and Z2 (Stothard et al., 1998). Our study revealed one isolate (SMM1) with a hybrid profile associated with Z3 and TcII. This result may corroborate the hypothesis that this isolate is the product of a

mixture of parasite populations, since the vector in wild environments may feed on several vertebrate hosts. This complexity was demonstrated in the State of Rio de Janeiro by Fernandes et al. (1999), who showed a preferential association of the two lineages of *T. cruzi* with different hosts. They suggest that the vector *T. vitticeps* is involved in the transmission cycle among mammals infected by lineage 2 in the municipality of Teresópolis, and in the transmission cycle of primates in municipality of Silva Jardim. The hybrid profile found in these samples may indicate a possibility that the vector *T. vitticeps* does not only participate in the wild cycle of the disease.

The main purpose of typing of isolates of *T. cruzi* is to identify strains with different epidemiological and/or clinical characteristics of Chagas disease. Our results corroborate other descriptions in the literature, and contribute to the knowledge and records of the profile of some additional wild isolates of *T. cruzi* in regions not yet affected by the disease. Added to the complexity observed between the isolates is the finding that the Z3 profile is divided into two groups, called Z3a and Z3b (Mendonça et al., 2002). Our laboratory is interested in investigating whether such a dichotomy occurs among the Z3 isolates obtained from *T. vitticeps* in this area of study.

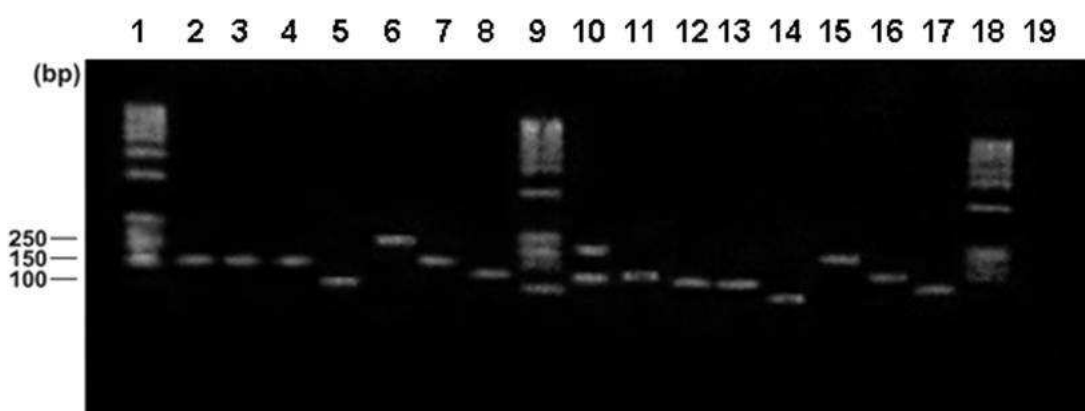


Fig. 4. PCR Multiplex – Mini-exon. The gel of agarose for electrophoresis was amplified using isolates of *Trypanosoma cruzi* of reference that possess approach bands of TCI, compared to TCII, Z3 and *Trypanosoma rangeli* and with *T. cruzi* sylvaticus isolates from Rio de Janeiro. The isolates was performed using 25 ng of genomic DNA extracted using the phenol-chloroform method. Five primers were used: for Tc1 (5'-TTG CTC GCA CAC TCG GCT GCAT-3'), for Tc2 (5'-ACA CTT TCT GTG GCG CTG ATC G-3'), for Z3 (CCG CGW ACA ACC CCT MAT AAA AAT G-3'), for Tr (CCT ATT GTG ATC CCC ATC CCC ATC TTC G-3'), and for the mini-exon (5' TAC CAA TAT AGT ACAGAA ACT G-3'). Lane 1. Molecular weight marker (100bp DNA ladder), 2. SMM98, 3. SMM36, 4. SMM82, 5. *T. rangeli*, 6. CL Brener, 7. DM28c, 8. JJ, 9. Molecular weight marker (100bp DNA ladder), 10. SMM1, 11. SMM10, 12. SMM53, 13. SMM88, 14. *T. rangeli*, 15. CL Brener, 16. DM28c, 17. JJ, 18. Molecular weight marker (100bp DNA ladder), 19. negative control (no DNA added). bp = base pairs.

3.3 Proteolytic enzymes

Despite the existing knowledge of this flagellate genome and its main families of proteins, little is known about these parasites isolated from triatomines captured in the field, as well *T. cruzi* in mammals of wild origin. Proteolytic enzymes are reported to play an important role in determining the virulence of these microorganisms.

Proteases are essential for all life forms. They are involved in a multitude of physiological reactions, ranging from simple digestion of proteins for nutritional purposes, to highly-regulated metabolic cascades (e.g. proliferation and growth, differentiation, signaling and death pathways), and are essential for homeostatic control in both prokaryote and eukaryote cells (Rao et al., 1998). Proteases are also essential molecules in viruses, bacteria, fungi and protozoa, for their colonization, invasion, dissemination and evasion of host immune responses, mediating and sustaining the infectious disease process. Collectively, proteases participate in different steps of the multifaceted interaction events between microorganism and host structures, being considered as virulent attributes. Consequently, the biochemical characterization of these proteolytic enzymes is of interest not only for understanding proteases in general, but also for understanding their roles in microbial infections, and thus, their use as targets for rational chemotherapy of microbial diseases (Santos, 2010) (dos Santos, 2011).

Proteases are subdivided into two major groups, depending on their site of action: exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino (NH₂) or carboxyl (COOH) termini of the proteinaceous substrate, whereas endopeptidases cleave peptide bonds within a polypeptide chain. Based on their site of action at the NH₂ terminal, the exopeptidases are classified as aminopeptidases, dipeptidyl peptidases or tripeptidyl peptidases that act at a free NH₂ terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide or a tripeptide, respectively. Carboxypeptidases or peptidyl peptidases act at the COOH terminal of the polypeptide chain and liberate a single amino acid or a dipeptide (which can be hydrolyzed by the action of a dipeptidase). Carboxypeptidases can be further divided into three major groups: serine, metallo and cysteine carboxypeptidases, based on the functional group present at the active site of the enzymes. Similarly, endopeptidases are classified according to essential catalytic residues at their active sites in: serine, metallo, glutamic, threonine, cysteine and aspartic endopeptidases. Conversely, there are a few miscellaneous proteases that do not precisely fit into the standard classification (dos Santos, 2010, 2011).

Cysteine peptidases from parasitic protozoa have been characterized as factors of virulence and pathogenicity in several human and veterinary diseases. *T. cruzi* contains a major cysteine peptidase named cruzipain (also known as cruzain or GP57/51), which is present in different developmental forms of the parasite, although at variable levels (Dos Reis et al., 2006). Cruzipain is a papain-like peptidase that shares biochemical characteristics with both cathepsin L and cathepsin B (Cazzulo et al., 1990b). Cysteine peptidases have already been detected in many species of Trypanosomatidae, and are regarded as essential for the survival of several parasitic protozoa. The enzyme has been shown to be lysosomal, and is located in an epimastigote-specific pre-lysosomal organelle called the 'reservosome', which contains proteins that are digested during differentiation to metacyclic trypomastigotes (Soares et al., 1992). Some authors have suggested a second location of enzyme isoforms in the plasma membrane, associated with a glycosylphosphatidylinositol (GPI) anchor (Elias et al., 2008). These isoforms were present in epimastigotes, amastigotes and trypomastigotes, and reacted with polyclonal anti-cruzipain sera, thereby becoming an immunodominant antigen that is recognized by the sera of human patients with chronic Chagas disease (Martínez et al., 1991). Recently, the peptidase expression analysis of fresh field sylvatic isolated strains of *T. cruzi* showed a heterogeneous profile of cysteine proteolytic activities in the main phylogenetic groups TCI and TCII (Fampa et al., 2008).

Gomes et al (2009) investigated the production of peptidases, especially cruzipain, as well as the protein surface distribution in four newly sylvatic isolates of *T. cruzi* belonging to the Z3 genotype.

3.4 Proteolytic profile of *T. cruzi* isolates from Rio de Janeiro

The differences in peptidase expression between TCI and TCII phylogenetic groups have recently been investigated. Since *T. cruzi* isolates from sylvatic triatomines were included in the third phylogenetic group, named Z3, our investigation contributes to investigate the expression of surface polypeptides and the major cysteine peptidase from the Z3 parasite population, thereby furthering understanding on the genetic variability in the pathogenesis of Chagas disease. In this context, we carried out an identification of the protein profile and peptidase from epimastigotes (replicative forms of this parasite) of sylvatic isolates of *T. cruzi* (classified as Z3) from triatomines captured in Santa Maria Madalena (SMM) in the State of Rio de Janeiro. The separation of soluble whole proteins revealed a different protein profile, with approximately 35 polypeptides presenting apparent molecular masses from 118 to 25 kDa in all the samples. The proteolytic activity was determined by zymograms analysis of all the samples, using SDS-polyacrylamide gel electrophoresis containing gelatin as substrate. Our main results demonstrate a major band of 45 kDa sensible to E-64, a powerful cysteine peptidase inhibitor, in all the samples. In order to confirm this data, western blotting was performed using the anti-cruzipain polyclonal antibody. These findings showed a strong polypeptide band with an apparent molecular mass between 40 and 50 kDa in all the sylvatic isolates: SMM10; SMM53; SMM88 and SMM98 respectively and also Dm28c (Figure 5).

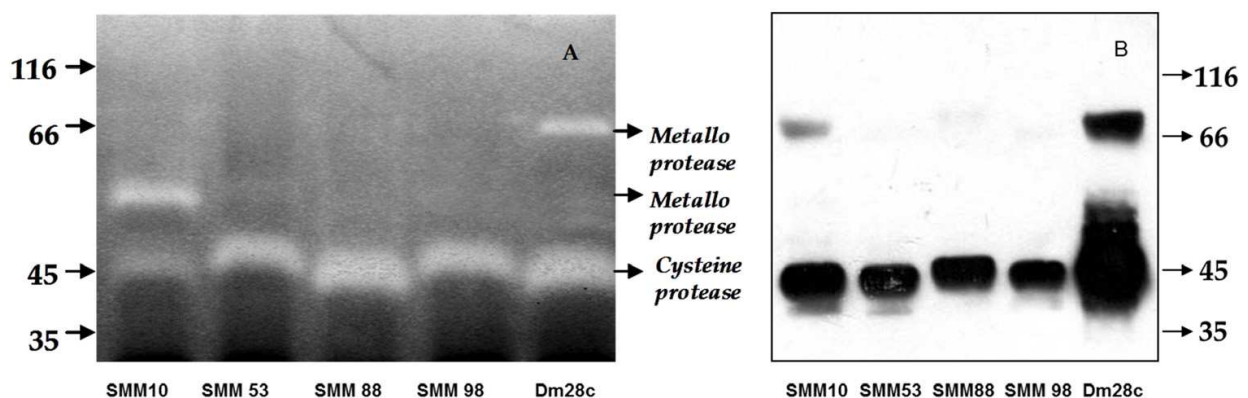


Fig. 5. A - Gelatin-SDS-PAGE showing the proteolytic activity profiles of *T. cruzi* sylvatic isolates. Parasites (SMM10, SMM53, SMM88, SMM98, and Dm28c) grown for 7 days were harvested and lysed by SDS. The gel was incubated in 50 mM sodium phosphate buffer, pH 5.5, supplemented with 2 mM DTT for 40 h at 37°C; B- Western blotting showing the reactivity of cellular polypeptides of *T. cruzi* sylvatic isolates with the anti-cruzipain polyclonal antibody. Numbers on the left indicate the relative molecular mass markers, expressed in kilodaltons.

These results show the presence of a main cysteine peptidase, cruzipain, in the sylvatic isolates of *T. cruzi* from Santa Maria Madalena, in the State of Rio de Janeiro (Gomes et al., 2009). We also observed another gelatinolytic activity of 66 kDa that was recognized by the anti-cruzipain antibody, probably a cruzipain isoform; since cruzipain is a high mannose-

type glycoprotein containing about 10% carbohydrate, its molecular mass can be estimated from the sequence, considering two high-mannose oligosaccharide chains, as about 40 kDa. However, this enzyme can present anomalous behavior in SDS-PAGE, yielding apparent molecular mass values of 35 to 60 kDa depending on the experimental conditions. The cysteine peptidases from parasites, including *T. cruzi*, have proven to be valuable targets for chemotherapy. Due to the biological importance of cruzipain in the life cycle of *T. cruzi*, many studies have sought to build specific inhibitors against the active core of this enzyme, in order to obtain a new drug capable of providing protection against human infection by *T. cruzi*.

4. Conclusion

Trypanosoma cruzi shows considerable heterogeneity among populations isolated from sylvatic and domestic cycles. Despite of knowledge concerning the genome of these flagellated organisms and their main protein families, very little is known about these parasites isolated from triatomine bugs captured from field, as well as *T. cruzi* extracted from sylvatic mammals. In this context, we do hereby highlight the importance of molecular studies on *T. cruzi* sylvatic isolates collected by blood culture from vertebrate hosts and/or from triatomine vectors, *Triatoma vitticeps*, in Triunfo location, 2nd district of Santa Maria Madalena city, Northern region of Rio de Janeiro State, Brazil. The results of our investigations with *T. cruzi* samples isolated from sylvatic triatomine insects revealed that these parasites belong to a phylogenetic group called ZIII, and proteolytic analyzes evidenced the presence of a key peptidase cysteine, cruzipain, in all samples of sylvatic *T. cruzi* isolates from Santa Maria Madalena - Rio de Janeiro (Brazil), which was confirmed by anti-cruzipain antibody recognition. Taken together, our results can corroborate in understanding the role of proteolytic enzymes in determining the virulence of these microorganisms, as well as genetic variability of Z3 population in Chagas disease pathogenesis.

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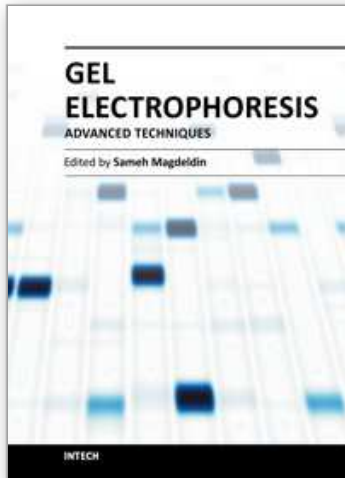
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