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BRAZILIAN ISOLATES OF *TRYPANOSOMA CRUZI* FROM HUMANS AND TRIATOMINES CLASSIFIED INTO TWO LINEAGES USING MINI-EXON AND RIBOSOMAL RNA SEQUENCES

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Abstract. Traditional molecular and biochemical methods, such as schizodeme analysis, karyotyping, DNA fingerprinting, and enzyme electrophoretic profiles, have shown a large variability among *Trypanosoma cruzi* isolates. In contrast to those results, polymerase chain reaction (PCR) amplification of sequences from the 24Sα ribosomal RNA gene and from the mini-exon gene nontranscribed spacer indicated a dimorphism among *T. cruzi* isolates, which enabled the definition of two major parasite lineages. In the present study, 86 *T. cruzi* field stocks (68 isolated from humans with defined presentations of Chagas' disease and 18 from triatomines) derived from four Brazilian geographic areas were typed by the PCR assay based on the DNA sequences of the mini-exon and 24Sα rRNA genes. These stocks were ordered into the two major *T. cruzi* lineages. Lineage 1 was associated mainly with human isolates and lineage 2 with the sylvatic cycle of the parasite.

Transmission of the protozoan parasite *Trypanosoma cruzi* involves invertebrate vectors (triatomines) and wild mammals, including marsupials, carnivores, rodents, and other animals, which define the sylvatic cycles of the parasite. Social and ecologic factors, such as domestic animals acting as reservoirs of the trypanosome and domiciliation of the vector, bring humans into contact with *T. cruzi*. This peridomestic cycle is the most important in relation to Chagas' disease because it exposes 90 million persons to the risk of infection.[1]

The different biologic, epidemiologic, and clinical patterns of the *T.cruzi*-human interaction have stimulated a major effort to determine biochemical or molecular markers that correlate with specific features. Based on enzyme electrophoretic profiles, three distinct groups of *T. cruzi* isolates were identified (zymodemes), which corresponded to the sylvatic (zymodeme 1 and less commonly, zymodeme 3) or domestic (zymodeme 2) cycles.[2–4] Further isoenzyme analysis indicated a greater genetic heterogeneity among *T. cruzi* isolates.[5] This large variability among parasite populations was also observed in restriction-fragment-length polymorphism in the mitochondrial DNA (schizodeme analysis), nuclear DNA fingerprinting, or karyotyping studies.[6–10]

In contrast to the diversity suggested by traditional techniques, PCR amplification of sequences from the 24Sα ribosomal RNA (rRNA) gene and from the mini-exon intergenic region indicated a clear dimorphism among *T. cruzi* isolates.[11–13] An examination of 88 *T. cruzi* stocks derived from humans, wild mammals, and triatomines from different countries of South America (Brazil, Argentina, Chile, Bolivia, and Venezuela) by mini-exon gene and 24Sα rRNA typing approaches, and randomly amplified polymorphic DNA analysis further defined two major parasite lineages with high phylogenetic divergence.[13] More recent observations indicated that the promoter regions of the rRNA cistron of *T. cruzi* show specific features and functional activities that corroborate the definition of the two major groups, especially when one considers that for many eukaryotes the rRNA promoter activity is strongly species-selective.[14,15]

To provide evidence of a possible association of epidemiologic parameters of *T. cruzi* isolates with the two clearly defined lineages, the mini-exon and/or 24Sα rRNA typing method were applied to 86 field stocks (68 isolated from humans and 18 from triatomines) derived from four Brazilian geographic areas. All isolates were clustered into the two major groups and data are suggestive of a preferential association of lineage 1 to human isolates and lineage 2 to the sylvatic cycle of the parasite.

MATERIALS AND METHODS

Endemic regions, inhabitant selection, and vector capturing. The studied areas, Amazonas (north), Paraiba (northeast), Piaui (northeast), and Minas Gerais (southeast) are shown in Figure 1. The individuals from these regions were selected after a positive serologic diagnosis for Chagas' disease obtained by indirect immunofluorescence using epimastigotes and an ELISA using an epimastigote cytosolic fraction.[16–18] In Piaui, *Triatoma braziliensis* and *Triatoma pseudomaculata* were manually collected in the vicinities of human dwellings, taxonomically classified, and their intestinal content was examined microscopically for the presence of flagellates. Only the former species was shown to be infected with *T. cruzi*. In the Amazon region, *Rhodnius brethesi* were captured using a light trap in the forest environment and subjected to the same procedures described.

Clinical examination, xenodiagnosis, and hemoculture. Seropositive individuals, after signing an informed consent form that was previously approved by the Ethical Committee of FIOCRUZ (Ministry of Health, Rio de Janeiro), were subjected to a careful clinical examination followed by conventional electrocardiography and barium-contrasted radiographs if any symptom suggestive of esophagus involvement was described. Xenodiagnosis was performed using third-

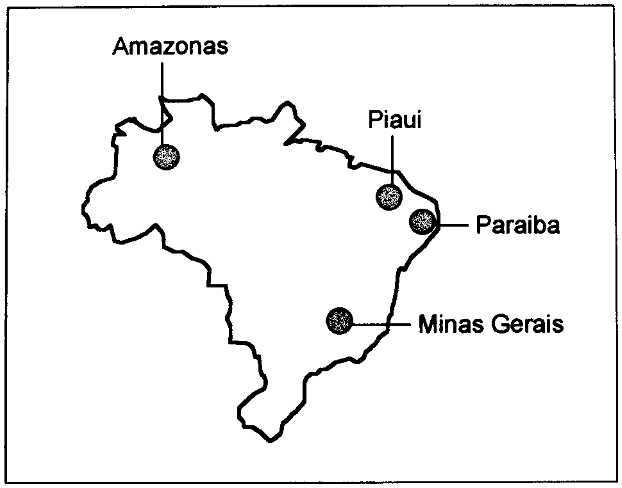

FIGURE 1. Map of Brazil showing the studied areas.

instar larvae of both *Panstrongylus megistus* and *Triatoma infestans.* Twenty nymphs were allowed to feed on each patient for 30 min. Microscopic examination of intestinal content was carried out 45 days after feeding. For hemoculture, approximately 30 ml of blood was collected in tubes containing heparin and centrifuged for 10 min at 4,000 × *g*. One volume of liver infusion tryptose (LIT) medium was added to the blood cellular components after discarding the supernatant.[19] The samples were centrifuged again and the cell pellet was resuspended in 20 ml of LIT medium and equally divided into five culture tubes. The tubes were incubated at 28°C. The cultures were microscopically examined on days 45, 60, 90, and 120 after blood collection.

Parasite culture and extraction of DNA. *Trypanosoma cruzi* stocks were cultured in LIT medium at 28°C for two weeks. Approximately 1 ml of growing culture was centrifuged at 13, 000 × *g* and the cells were resuspended in 200 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) and incubated at 56°C for 2 hr with 100 μg/ml of proteinase K and 0.5% sodium dodecyl sulfate. The lysate was extracted with phenol:chloroform (1:1) and the DNA was precipitated after the addition of sodium acetate and ethanol.

Mini-exon intergenic region amplification. Amplification of part of the intergenic region of the mini-exon gene was achieved using a pool of three oligonucleotides (Figure 2A). Two oligonucleotides, derived from a hypervariable region were used as upstream primers (TC1 - 5'-GTGTCCGCCACCTCCTTCGGGCC-3' and TC2 - 5'-CCTG CAGGCACACGTGTGTGTG-3') and a common downstream oligonucleotide, corresponding to sequences present in both *T. cruzi* lineages (TC - 5'-CCCCCCTCCCA-GGCCACACTG-3'). The PCRs were carried out using 1 ng of genomic DNA as template, 100 pmoles of each primer, 100 μM dNTPs, 1 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in the buffer recommended by the manufacturer. The amplification procedure consisted of denaturing at 94°C, annealing at 55°C, and extension at 72°C. Every step was done for 30 sec and 27 cycles were carried out. Amplified products were analyzed by agarose gel (2.0%) electrophoresis and UV visualization after staining with ethidium bromide.[13]

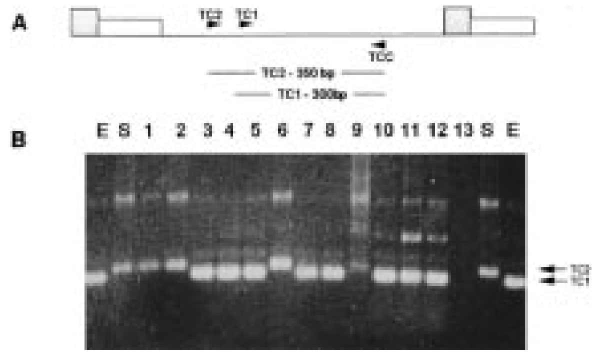

FIGURE 2. Mini-exon gene typing assay. **A,** mini-exon gene scheme. The exon is the hatched box and the intron is presented as the open box. The nontranscribed spacer or intergenic region is represented by the line. The **arrowheads** correspond to the primers used in the polymerase chain reaction (PCR) (TC1 - lineage 1, TC2 - lineage 2, and TCC - *Trypanosoma cruzi* common primer). The size of the expected PCR products are shown: TC1 = 300 basepairs (bp) and TC2 = 350 bp. **B,** agarose gel electrophoresis of PCR-amplified products. Standard stocks: E = Esmeraldo - lineage 1; S = Silvio X 10 - lineage 2. Lanes 1–12, MT 01, MT 03, MT 05, MT 07, MT 08, MT 16, MT 18, MT 20, MT 27, MT 81, MT 82, and MT 83; lane 13, negative control (no DNA was added to the PCR). The **arrows** show the sizes of the PCR products from lineage 1 and lineage 2.

Ribosomal RNA gene amplification. The PCR amplification of a divergent domain of the 24Sα ribosomal RNA gene was achieved with two primers (Figure 3A): D71 - 5'-AAGGTGCGTCGACAGTGTGG-3' and D72 -5'-TTTTCA-GAATGGCCGAACAGT-3' following a previously described protocol.[11] The reaction products were subjected to electrophoresis on a 7.5% polyacrylamide gel, stained with ethidium bromide, and visualized under UV light.

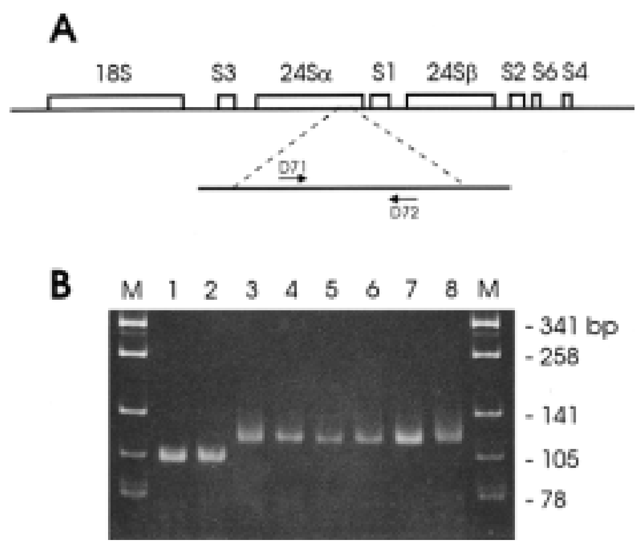

FIGURE 3. Polymerase chain reaction typing assay based on the 24Sα rRNA gene. **A,** organization of the ribosomal RNA cistron of *T. cruzi*.[24] The sizes of rRNA genes are not proportional. The localization of primers D71 and D72 that amplify part of the 24Sα rRNA gene are indicated. **B,** polyacrylamide gel electrophoresis of amplified products. Lanes 1 and 2, MT 01 and MT 03 - lineage 2; lanes 3–8, MT 05, MT 06, MT 07, MT 08, MT 09, and MT 10 - lineage 1; lane M, molecular weight markers. bp = basepairs.

TABLE 1
Molecular typing of *Trypanosoma cruzi* isolates from humans, geographic origin, clinical forms, and lineage classification

Origin	Isolates	Clinical manifestations	Lineage	Origin	Isolates	Clinical manifestations	Lineage
Amazonas	MT01	Indeterminate	2	Piaui	MT52	Indeterminate	1
	MT03	Acute phase	2		MT53	Indeterminate	1
					MT55	Indeterminate	1
Paraiba	MT05	Indeterminate	1		MT56	Cardiopathy and digestive form	1
	MT06	Cardiopathy	1		MT58	Cardiopathy	1
	MT07	Indeterminate	1		MT59	Indeterminate	1
	MT08	Indeterminate	1		MT60	Cardiopathy and digestive form	1
	MT09	Cardiopathy	1		MT61	Indeterminate	1
	MT10	Cardiopathy	1		MT62	Indeterminate	1
	MT11	Cardiopathy	1		MT63	Cardiopathy	1
	MT12	Indeterminate	1		MT64	Cardiopathy	1
	MT13	Cardiopathy	1		MT66	Indeterminate	1
	MT14	Indeterminate	1		MT67	Indeterminate	1
					MT68	Cardiopathy	1
Piaui	MT17	Cardiopathy	1		MT72	Indeterminate	1
	MT18	Indeterminate	1		MT77	Indeterminate	1
	MT19	Cardiopathy	1		MT78	Indeterminate	1
	MT20	Indeterminate	1		MT79	Cardiopathy	1
	MT21	Indeterminate	1				
	MT24	Cardiopathy	1	Minas Gerais	MT81	Indeterminate	1
	MT25	Cardiopathy	1		MT82	Cardiopathy	1
	MT30	Cardiopathy	1		MT83	Indeterminate	1
	MT32	Cardiopathy	1		MT84	Cardiopathy	1
	MT33	Indeterminate	1		MT85	Cardiopathy	1
	MT34	Indeterminate	1		D143*	Cardiopathy and digestive form	2
	MT38	Cardiopathy	1		D207*	Indeterminate	2
	MT40	Indeterminate	1		A138*	Cardiopathy and digestive form	1
	MT41	Cardiopathy	1		B147*	Cardiopathy	1
	MT44	Cardiopathy	1		B167*	Cardiopathy	1
	MT45	Cardiopathy and digestive form	1		C182*	Cardiopathy and digestive form	1
	MT46	Indeterminate	1		C222*	Indeterminate	2
	MT47	Indeterminate	1		D226*	Indeterminate	1
	MT48	Cardiopathy	1		231*	Cardiopathy and digestive form	2
	MT49	Cardiopathy	1		Basileu*	Acute phase	1
	MT50	Cardiopathy	1		JM*	Acute phase	1

RESULTS

Previous analysis of a wide sample of *T. cruzi* strains and clones from vertebrate and invertebrate hosts showed a strict correlation between the genotypes defined by two nuclear markers, the mini-exon gene and the ribosomal RNA gene, which allowed the definition of two *T. cruzi* lineages.[13] However, in that study, an eventual association between epidemiologic parameters of the isolates and a given genotype was not investigated. To survey trypanosome strains present in the domestic cycle, and in regions where human habitations encroach on forested areas, we obtained 86 samples of *T. cruzi* from Amazonas (6), Paraiba (10), Piaui (49), and Minas Gerais (21). These parasites include 68 samples from humans with well-documented Chagas' disease at different stages and with distinct pathologies (Table 1) and 18 stocks from reduviid vectors (Table 2). The DNA extracted from these samples was subjected to the PCR assays that distinguish two groups of *T. cruzi*.

TABLE 2
Molecular typing of *Trypanosoma cruzi* isolates from triatomines, geographic origin, and lineage classification

Origin	Isolate	Vector	Lineage
Amazonas	AM01	*Rhodnius brethesi*	2
	AM02	*Rhodnius brethesi*	2
	AM03	*Rhodnius brethesi*	2
	AM04	*Rhodnius brethesi*	2
Piaui	MT15	*Triatoma brasiliensis*	1
	MT16	*Triatoma brasiliensis*	2
	MT22	*Triatoma brasiliensis*	1
	MT23	*Triatoma brasiliensis*	1
	MT26	*Triatoma brasiliensis*	1
	MT27	*Triatoma brasiliensis*	2
	MT28	*Triatoma brasiliensis*	1
	MT29	*Triatoma brasiliensis*	1
	MT31	*Triatoma brasiliensis*	1
Minas Gerais	1004*	*Triatoma infestans*	2
	1006*	*Triatoma infestans*	2
	1009*	*Panstrongylus megistus*	2
	1018*	*Panstrongylus megistus*	2
	1023*	*Panstrongylus megistus*	1

* Cryopreserved stocks.

First, the mini-exon typing assay was performed on all 86 samples with three primers (a *T. cruzi* common primer -TC, and two lineages specific primers - TC1 and TC2 - Figure 2A) in a single PCR, which results in two possible amplified products of 300 basepairs (bp) (lineage 1) and 350 bp (lineage 2) that can be distinguished by agarose gel electrophoresis (representative examples are shown in Figure 2B). Seventy samples were clustered as lineage 1 and 16 as lineage 2 by this criterion.

Second, amplification of 24Sα rRNA gene sequence was performed in 23 samples by PCR using two primers localized at the 3' portion of the gene (Figure 3A). The possible results of this assay are DNA fragments of 125 bp (lineage 1) and 110 bp (lineage 2) respectively, which can be separated by polyacrylamide gel electrophoresis (representative examples are shown in Figure 3B). By this assay, 21 samples were classified as *T.cruzi* lineage 1 and 2 isolates assigned as lineage 2; the remaining 61 samples were not tested by the rRNA gene assay since in a previous report we have shown a strong correlation between the mini-exon and rRNA lineage typing.[13]

The genotypes of the 68 human isolates (57 recently isolated field samples and 11 cryopreserved stocks) are described in Table 1. Sixty-two (91%) isolates were classified as lineage 1 and six stocks were typed as lineage 2.

In Table 2, data regarding the typing of 18 stocks isolated from four different species of triatomines are shown. *Trypanosoma cruzi* lineage 2 was found in 10 of 18 reduviids and the isolates from the remaining eight bugs belonged to lineage 1. The finding of both lineages in *Triatoma brasiliensis* and *P. megistus* indicates that both vectors have the potential to transmit the two *T. cruzi* genotypes.

DISCUSSION

Two independent nuclear markers, which have proven to be useful in the identification of two lineages in *T. cruzi* taxon, were used to type field isolates and cryopreserved stocks of the protozoan to search for a possible association between the genotypes with an eventual epidemiologic parameter of American trypanosomiasis.

The field stocks originated from four distinct geographic areas in Brazil, and were isolated from triatomines or humans by xenodiagnosis or hemoculture. Data in Table 1 indicate a strong tendency of human isolates (62 of 68; 91%) to belong to *T. cruzi* lineage 1. Because the field stocks from humans were isolated either by hemoculture or xenodiagnosis, these two procedures of parasite amplification should be considered as biologic filters. The use of a single triatomine species in xenodiagnosis may select specific populations of the protozoan with the possibility of losing strains that are poorly adapted to the vector. Indeed, previous studies indicate differences in the growth and development of *T. cruzi* clones in triatomine vectors.[20] We have used two triatomine species (*P. megistus* and *Triatoma infestans*) to minimize possible selection of parasite subpopulations. Data presented in Table 2 indicate that both vectors are able to transmit the two *T. cruzi* lineages.

While Paraiba, Piaui, and Minas Gerais are well known endemic regions with a high prevalence of Chagas' disease, Amazonas State is associated with sporadic reports of human cases of this disease. *Trypanosoma cruzi* is an enzootic parasite in the Brazilian Amazon, affecting wild animals and insect vectors, and the human cases can be attributed to either accidental contact with infected wild triatomines or to human migration from other Brazilian endemic areas of Chagas' disease. The human samples derived from the Amazonas State (two isolates) were obtained from one acute case of Chagas' disease and from one asymptomatic patient. This latter individual was selected after a serologic survey that demonstrated 13.2% positivity (170 of 1,286) for anti-*T. cruzi* antibodies in the general population.[16] Of the 170 positive patients, 82 were subjected to xenodiagnosis and *T. cruzi* was isolated from the fed triatomines in two cases.[16] One of the isolates was lost during cultivation; however, the other was typed as lineage 2 (isolate MT01, Table 1). The low rate of *T. cruzi* isolation from chagasic seropositive individuals in Amazonas could be explained by a low parasitemia.

Based on data regarding the positivity of xenodiagnosis and PCR amplification of the variable region of the kinetoplast DNA minicircle molecule for diagnostic purposes, it seems that lineage 1 presents a higher parasitemia in humans than lineage 2. Indeed, studies performed in individuals seropositive for Chagas' disease in Paraiba, Piaui, and Minas Gerais indicate that xenodiagnosis was positive in 13%, 34%, and 46%, respectively, while PCR was positive in 45% (Paraiba), 60% (Piaui) and 97% (Minas Gerais).[16, 18, 21] Regarding the Amazonas State, where a higher isolation rate of *T. cruzi* stocks by xenodiagnosis due to the seroprevalence (12%) was expected, the PCR was positive in 10% of the cases and correlated with the low isolation rate of the protozoa by the xenodiagnosis procedure.[16]

Another possibility for the low rate of *T. cruzi* isolation from the seropositive patients in Amazonas might be the fact that xenodiagnosis was performed with *Triatoma infestans* and *P. megistus.* In fact, the vector to which the Amazon isolates may have been adapted is *Rhodnius brethesi,* which is found exclusively in the sylvatic environment, and all *T. cruzi* stocks isolated from this insect were genotyped as lineage 2 (Table 2).[22]

In the present study, seven of nine *T. cruzi* stocks isolated from *Triatoma brasiliensis* captured nearby human dwellings in Piaui were typed as belonging to lineage 1(Table 2), which was also observed in all human patients (30 samples) in that region (Table 1). In Minas Gerais State, infected *P. megistus* and *Triatoma infestans* were detected, harboring predominantly *T. cruzi* lineage 2 (Table 2). Although the two samples of captured *Triatoma infestans* contained *T. cruzi* lineage 2, this vector, considered to have a strong adaptation to artificial ecotopes, has been found infected with lineage 1 in samples isolated in Brazil (Rio Grande do Sul State) and in Bolivia (Tupiza) (Souto RP, unpublished data).[23]

Analysis of previous data suggest that *T. cruzi* lineage 2 can be associated with the sylvatic cycle since this genotype was predominantly found in parasites isolated from natural reservoirs such as opossums, armadillos, rodents, and other animals.[13] Furthermore, a correlation between the lineage 2 with *T. cruzi* zymodeme 1, which has been found to be the main zymodeme circulating in the sylvatic transmission cycle, have been suggested.[2, 3, 13] The two human and four insect samples of *T. cruzi* from Amazonas State were typed as lineage 2, providing further evidence that this genotype is involved in enzootic cycles of the parasite. With uncontrolled deforestation, one characteristic of emerging districts in the Amazon region, wild animals will of necessity be driven into other areas, changing some ecologic peculiarities of the triatomines, which are forced to adapt to alternative food sources in the peridomiciliary areas. In this case, humans may act as accidental hosts in the maintenance of the epidemiologic cycle. Considering the genotype presented by the majority of other human isolates in this study (91%) from Minas Gerais, Paraiba, and Piaui, which can be classified as lineage 1, we postulate that population migration probably does not contribute to the appearance of human cases in the area that we have studied in the Amazonas State.

Although there is a strong correlation of lineage 1 with human hosts, it is not possible to make any conclusion about the predictive value for clinical presentations in Chagas' disease since our sample is derived mainly from individuals with the indeterminate and cardiac form (Table 1). Further studies including more samples from other Latin America countries are being conducted to determine other epidemiologic and biologic features that could be associated with the two distinct *T. cruzi* lineages.

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