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Genetic Heterogeneity in *Trypanosoma cruzi* Strains From Naturally Infected Triatomine Vectors in Northeastern Brazil: Epidemiological Implications

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Eighteen Trypanosoma cruzi *strains isolated from naturally infected triatomines were studied genetically. The majority of the strains were from* Triatoma brasiliensis*, the principal vector of Chagas disease in the northeast of Brazil. Multilocus enzyme electrophoresis (MLEE) and randomly amplified polymorphic DNA (RAPD) analyses were used to investigate the genotypic diversity and the spread of the* T. cruzi *genotypes in different environments. MLEE clearly distinguished two distinct isoenzyme profiles, and RAPD analysis revealed 10 different genotypes circulating in rural areas. The strains could be typed as isoenzyme variants of the* T. cruzi *principal zymodeme Z1 (*T. cruzi *I). An effective program of epidemiological vigilance is required to prevent the spread of* T. cruzi *I strains into human dwellings.*

KEY WORDS: Chagas disease; MLEE; molecular epidemiology; RAPD; triatomines; *Trypanosoma cruzi*.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas disease, is mainly transmitted by contaminated feces of reduviid bugs of the subfamily Triatominae; strategies

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of control are directed against these insect vectors. The disease, being originally enzootic, cannot be included in the "eradicable diseases" (Silveira and Vinhaes, 1999). Transmission will continue in sylvatic environments among reservoirs and vectors; consequently, domiciliary transmission can also be reestablished from sylvatic foci. The control of Chagas disease is considered a high priority by Latin American governments. According to the World Health Organization, the disease affects more than 16 million people, and about 25% of the Latin American population (some 100 million people) is at risk of acquiring the infection (http//www.who.int/tdr/diseases/chagas/direction.htm; http//www.int/ctd/chagas/burdens.htm). Even after intradomiciliary control of *Triatoma infestans* populations, within the Southern Cone Initiative, new infestations arise through passive transport in clothes or belongings by people traveling from infested to noninfested dwellings (Dias *et al*., 2002).

T. brasiliensis and *T. pseudomaculata* are considered the principal Chagas disease vectors in semiarid zones of northeastern Brazil because of their genetic–ecological characteristics, widespread distribution, high natural infection levels, and ability to occupy domiciliary and sylvatic ecotopes (Costa *et al*., 1997; Costa and Marchon-Silva, 1998; Silveira and Vinhaes, 1999). The domiciliation of wild vectors, human habits, and migration are among the most important epidemiological features concerning the health authorities.

T. cruzi exists in nature as a complex of heterogeneous populations presenting a remarkable degree of intraspecific biogenetic diversity (Pacheco and Brito, 1999; Campbell *et al*., 2004). The circulation of such populations in domestic and sylvatic cycles involves humans, vectors, and animal reservoirs. Initial studies using isoenzyme analysis revealed three major zymodemes: Z1 and Z3 found in sylvatic cycles, associated with arboreal mammals such as opossums (Z1) and terrestrial animals such as armadillos (Z3), and Z2 in domestic environments, associated with human cases of the disease and domestic animals such as dogs and rodents (Miles *et al*., 1978). Subsequently, genetic diversity was found among distinct strains, indicating that they could hardly be clustered in a few groups (Tibayrenc and Ayala, 1988). Intraspecific heterogeneity at nuclear and/or mitochondrial DNA levels as revealed by different molecular markers has been reported, confirming the multiclonal structure of *T. cruzi* (Tibayrenc *et al*., 1986; Morel *et al*., 1986; Solari *et al*., 1992; Macedo *et al*., 1992; Steindel *et al*., 1993; Acosta *et al*., 2001) and probably having important clinical and epidemiological impact (Vago *et al*., 2000). Nowadays, *T. cruzi* strains are classified into two main groups: *T. cruzi* I and *T. cruzi* II, which include the former zymodemes Z1 and Z2, respectively (Momen, 1999). According to Brisse *et al*. (2000), *T. cruzi* II appears to be subdivided into five subgroups (IIa–IIe), clustering the former zymodeme Z3 in the subgroup IIa. Conversely, *T. cruzi* I seems to be undivided, and strains from this group are found in both domestic and sylvatic cycles. Evidence supporting genetic exchange (Gaunt *et al*., 2003) and the occurrence of multiple hybrid groups

in *T. cruzi* (Sturm *et al*., 2003) has been reported, explaining in part its genetic diversity.

During a 19-month period (November 2000–April 2002) of entomological surveillance, a total of 2307 specimens of *T. brasiliensis* were captured in several localities of rural areas of the Jaguaruana municipality in the state of Ceará, northeast region of Brazil (Sarquis *et al*., 2004). From that total, 85.7% of the specimens were found in peridomestic environments. Ratios of 15.7 and 10.7% of naturally infected triatomines were detected in the peri- and intradomiciles, respectively (Sarquis *et al*., 2004). The present paper reports the results of the genotypic study of *T. cruzi* strains isolated from *T. brasiliensis* and *T. pseudomaculata* in four rural areas of the Jaguaruana municipality. Multilocus enzyme electrophoresis (MLEE) and randomly amplified polymorphic DNA (RAPD) analyses were used to evaluate the genotypic heterogeneity of *T. cruzi* strains that circulate in vectors and the spread of the genotypes in the different environments.

MATERIALS AND METHODS

Localities and *T. cruzi* Isolates

The municipality of Jaguaruana (4°50′09″S, 37°46′48″W) is situated in the zone of the lower Jaguaribe River, in the state of Ceará, only 183 km from the capital (Fortaleza). Triatomines were captured in the intra- and peridomiciles of four localities (Lagoa Vermelha, Corrego Salgado, Pacatanha, and Currais do Felipe), considered rural areas of the Jaguaruana municipality (Fig. 1). The majority of the *T. cruzi* strains were isolated from *T. brasiliensis* (17/18) and from *T. pseudomaculata* (1/18). Table I summarizes all information regarding sample code, place of capture, vector, and ecotopes.

T. cruzi strains were isolated from triatomine feces and maintained in biphasic cultures (Novy–Nicolle–McNeal, NNN; Brain Heart Infusion, BHI; 10% Fetal Bovine Serum, FBS) at 28°C. The cell mass was prepared by washing the parasites twice by centrifugation at 700*g* in SE 1× (0.15 M NaCl/0.1 M EDTA, pH 8.0) for 10 min at 4°C. Three *T. cruzi* reference strains were used as controls: Dm28c strain (Z1, *T. cruzi* I), CL strain (Z2, *T. cruzi* II), and Can III strain (Z3, Miles *et al*., 1980; subgroup IIa, Brisse *et al*., 2000).

Multilocus Enzyme Electrophoresis (MLEE)

Isoenzyme analyses were performed on agarose gel according to protocols previously described (Miles *et al*., 1980; Pacheco *et al*., 1999; Brito *et al*., 2003). The following 10 enzymes were used: glucose 6 phosphate dehydrogenase (G6PDH, E.C. 1.1.44), glucose phosphate isomerase (GPI, E.C. 5.3.1.9), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), malate dehydrogenase (MDH, E.C. 1.1.1.37),

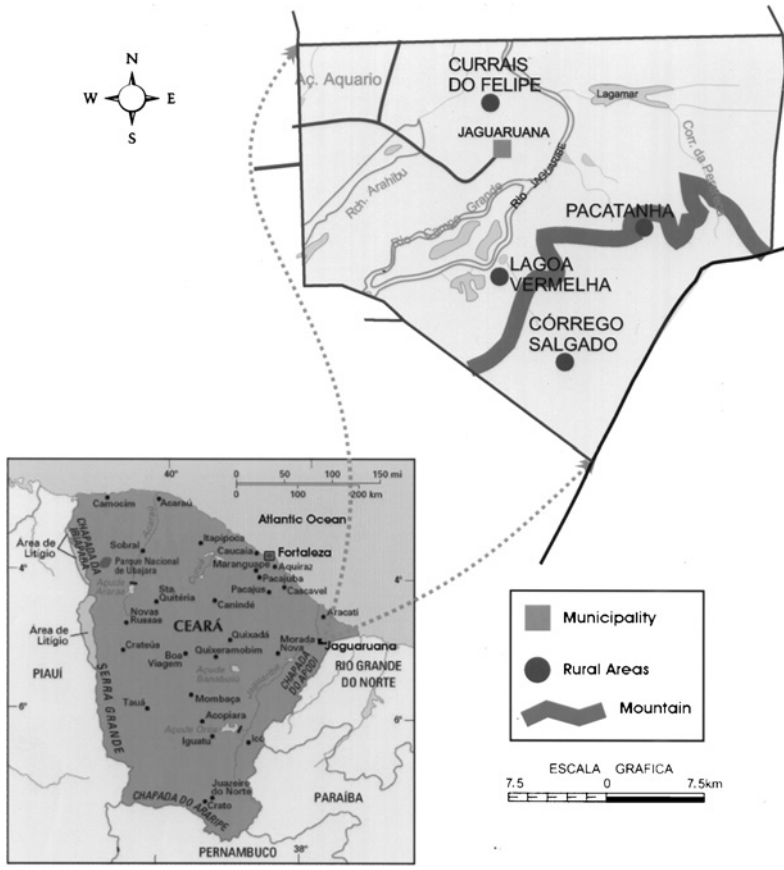

Fig. 1. The Jaguaruana municipality and sites where triatomines were captured. Inset: Location of study area within the Brazilian state of Ceará.

malic enzyme (ME, E.C. 1.1.1.40), nucleotidase (NH, E.C. 3.2.2.1), peptidase D (PEP-D, E.C. 3.4.13.9), peptidase 2 (PEP-2, E.C. 3.4.11.1), 6-phosphogluconate dehydrogenase (6PG, E.C. 1.1.44), and phosphoglucomutase (PGM, E.C. 2.7.5.1).

RAPD Analysis

Parasite genomic DNA was extracted on the basis of the protocol described by Gomes *et al*. (1995), using a commercial kit (DNA Genomic Prep; Amersham Pharmacia Biotech), followed by additional phenol–chloroform purifications. DNA concentrations and purity were further determined by agarose gel electrophoresis compared with known concentrations of a standard DNA. Six 10-base oligomer primers from a Ready-to-Go RAPD Analysis Kit (Amersham Pharmacia

Table I. Source of *T. cruzi* Strains

Code	Vector species	Locality	Ecotope
CE2	*T. brasiliensis*	Pacatanha	Intradomicile
CE3	*T. brasiliensis*	Corrego Salgado	Intradomicile
CE4	*T. brasiliensis*	Lagoa Vermelha	Intradomicile
CE8	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE14	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE21	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE25	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE28	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE32	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE33	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE49	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE51	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE53	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE54	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE56	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE58	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE60	*T. brasiliensis*	Currais do Felipe	Peridomicile
CE63	*T. pseudomaculata*	Currais do Felipe	Peridomicile

Biotech) were chosen, as they revealed different degrees of polymorphism and well-defined amplification under the manufacturer's instructions. Amplifications were performed on a programmable thermal cycler (GeneAmp PCR System 9600, Applied Biosystems) with the following cycles: 95°C for 2 min (1 cycle); 95°C for 1 min, 37°C for 1 min, 72°C for 2 min (35 cycles), and a final extension at 72°C for 10 min (1 cycle). A negative control (all reaction components except DNA) was included in all experiments. The PCR products were subjected to electrophoresis in 2% agarose gel in 1× Tris-borate-EDTA buffer stained with ethidium bromide and recorded on a gel documentation system (UVP Biomaging System) with a UV light transilluminator. Well-defined bands ranging in size from 300 to 1500 base pairs (bp) were selected for phenetic analyses.

Phenetic Analysis

The isoenzyme and RAPD patterns were compared using Jaccard's coefficient of similarity to determine the proportion of mismatched bands. The matrix of binary data was transformed into a dendrogram using an UPGMA algorithm (unweighted pair-group method with arithmetic averages) (Sneath and Sokal, 1973) and NTSYS program (version 2.0, Exeter Software, Setauket, NY).

RESULTS

MLEE analyses using 10 enzymatic systems showed homogeneity among the *T. cruzi* strains. The majority of the strains analyzed (94.4%, 17/18) showed

Table II. Electromorphs (Alleles) Present in Each of the 18 *T. cruzi* and Three Reference Strains Studied

	Enzymes									
Isolates	GPI	IDH	PGM	ME	NH	PEPD	PEP2	G6P	MDH	6PG
CE2	1	2	4	1	1	2	3, 4	1	1	1
CE3	1	2	4	1	1	2	3, 4	1	1	1
CE4	1	2	4	1	1	2	3, 4	1	1	1
CE8	1	2	4	1	1	2	3, 4	1	1	1
CE14	1	2	4	1	1	2	3, 4	1	1	1
CE21	1	2	4	1	1	2	3, 4	1	1	1
CE25	1	2	4	1	1	2	3, 4	1	1	1
CE28	1	1	4	1	2	2	3	1	1	1
CE32	1	2	4	1	1	2	3, 4	1	1	1
CE33	1	2	4	1	1	2	3, 4	1	1	1
CE49	1	2	4	1	1	2	3, 4	1	1	1
CE51	1	2	4	1	1	2	3, 4	1	1	1
CE53	1	2	4	1	1	2	3, 4	1	1	1
CE54	1	2	4	1	1	2	3, 4	1	1	1
CE56	1	2	4	1	1	2	3, 4	1	1	1
CE58	1	2	4	1	1	2	3, 4	1	1	1
CE60	1	2	4	1	1	2	3, 4	1	1	1
CE63	1	2	4	1	1	2	3, 4	1	1	1
Z1	1	2	4	1	1	2	3	1	1	1
Z2	2, 3, 4	1	1,3	1	2	2	3, 4	2	1	2, 3
Z3	1	1	2	1	1	1	1	1	1	1

Note. Z1, Dm28c strain; Z2, CL strain; Z3, Can III strain (according to Miles *et al.,* 1980, the zymodeme Z3 is represented by the Can III strain).

similar electromorphic patterns, and only in one strain (CE28) were polymorphisms detected in the enzymes IDH, NH, and PEP-2 (Table II). Phenetic analyses have allowed the division of the strains into two clusters separated by a coefficient of similarity of 0.62. The first cluster was formed by the majority of the strains displaying 100% of similarity. The zymodeme Z1 (*T. cruzi* I) was included in this first phenetic group, sharing 92% of common characteristics. The second cluster was formed by the strain CE28, considered the most differentiated. No apparent association was found either with the reference strain representative of *T. cruzi* II (Z2) or with the Can III reference strain (0.40 of similarity index). The isolated strains could be typed as isoenzyme variants of the zymodeme Z1 (Fig. 2).

Genetic heterogeneity was detected in the strains by RAPD analyses. All conditions were controlled such that the concentration of reagents and cycling conditions were unaltered in all experiments. Of six decameric primers tested, three (Primer 2, 5′-GTTTCGCTCC-3′; Primer 3, 5′-GTAGACCCGT-3′; and Primer 4, 5′-AAGAGCCCGT-3′) revealed different degrees of intraspecific genetic polymorphism, exhibiting an unequivocal banding pattern for each *T. cruzi* strain (Fig. 3). Although polymorphisms were observed in number and size of amplified

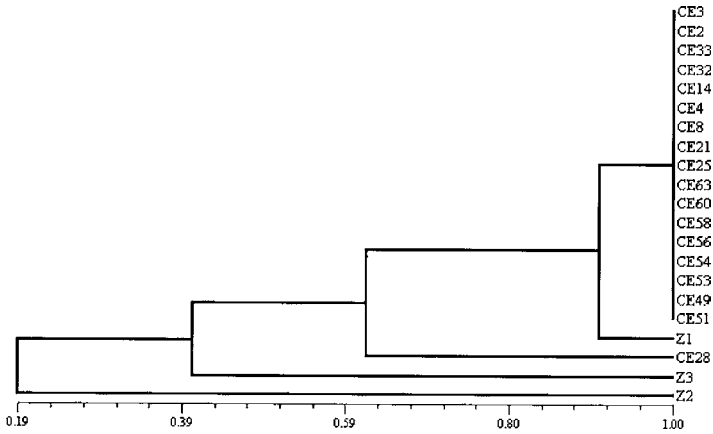

Fig. 2. Phenetic dendrogram based on isoenzyme patterns of the strains isolated from triatomines in Jaguaruana and *T. cruzi* reference strains, using Jaccard's coefficient of similarity. Z1, Dm28c strain; Z2, CL strain; Z3, Can III strain.

fragments, some conserved bands were detected among all isolated strains. One can observe a fragment of around 600 bp in all *T. cruzi* genomes after amplification with primer 2. Primer 4 was screened for its usefulness in distinguishing the three *T. cruzi* reference strains used as controls: CL (lane 7), Can III (lane 8), and Dm28C (lane 9). Comparison of amplification patterns and consensus cluster analysis (primers 2, 3, and 4) have separated the *T. cruzi* genotypes into three clusters (Fig. 4) that diverged by different coefficients of similarity (from 0.75 to 1.00). The RAPD pattern obtained from strain CE32 was considered to be from a more distant genotype, showing a coefficient of similarity of 0.63. The 17 strains found to be 100% similar by MLEE analyses could be further redistributed in their respective phenetic clusters. The *T. cruzi* strains CE3, CE4, CE8, CE21, and CE25 and the pair C14-CE56, displaying a coefficient of similarity of 1, were grouped in the first RAPD cluster. The zymodeme Z1 belongs to the same cluster, sharing 90% of common characteristics. The remaining strains showing 100% similarity (CE33, CE60, CE58, and the pair CE51-CE63) were grouped in a third cluster. An intermediate cluster (second cluster) was formed by the genotypes CE28 and CE53.

DISCUSSION

Many molecular markers have been used in population genetic studies. DNA-based analyses can provide information on evolutionary events, on phylogenetic relationships, and on the routes by which microorganisms spread. RAPD analysis, for instance, has the advantage of not requiring previous knowledge of the DNA

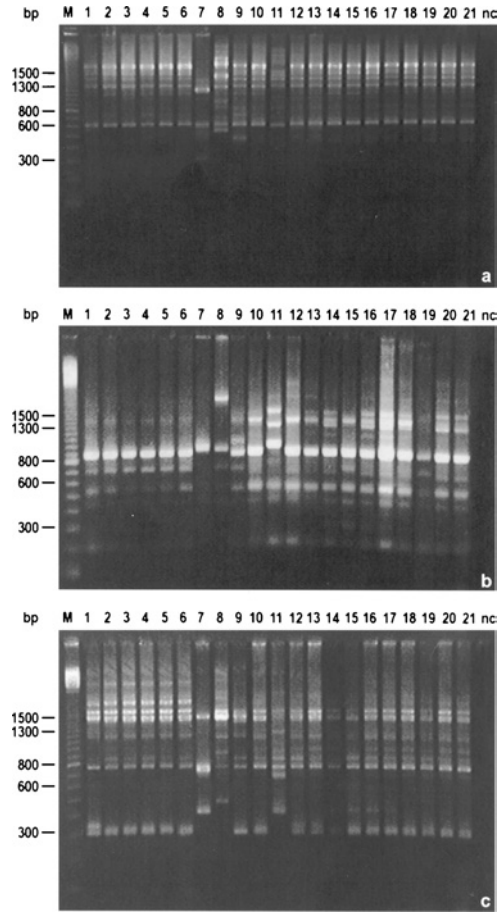

Fig. 3. Agarose gel electrophoresis showing RAPD profiles of the strains isolated from triatomines in Jaguaruana and *T. cruzi* reference strains after amplification with primers 2 (a), 3 (b), and 4 (c). The lanes contain: M, 100 bp DNA ladder; 1, CE2; 2, CE3; 3, CE4; 4, CE8; 5, CE21; 6, CE25; 7, CL strain; 8, Can III strain; 9, Dm28c strain; 10, CE14; 11, CE28; 12, CE32; 13, CE33; 14, CE49; 15, CE51; 16, CE53; 17, CE54; 18, CE56; 19, CE58; 20, CE60; 21, CE63; nc, negative control.

sequence, and because it can yield information on a large number of loci, it provides a more representative scenario of the genome than is possible with MLEE. The technique has been used in studies on taxonomy, species differentiation, and for genetic fingerprinting (Steindel *et al.*, 1993; Brisse *et al.*, 2000; Pacheco *et al.*, 2003).

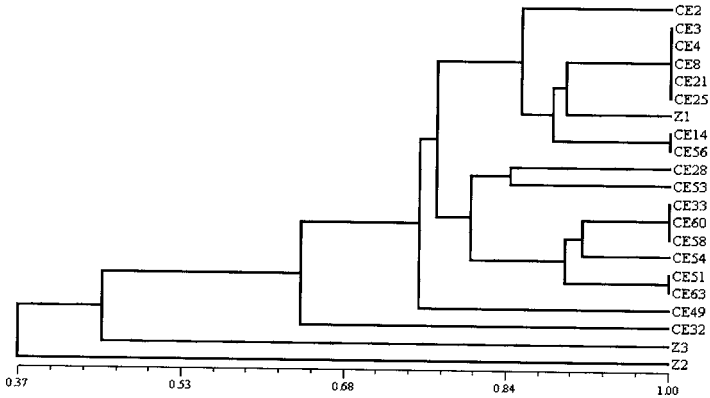

Fig. 4. RAPD-consensus phenetic dendrogram of *T. cruzi* strains from Jaguaruana and reference strains using Jaccard's coefficient of similarity. Z1, Dm28c strain; Z2, CL strain; Z3, Can III strain.

In the present paper we report the genotypic analysis of *T. cruzi* strains isolated from naturally infected triatomines using two distinct molecular markers. All strains were unequivocally typed by isoenzymes as *T. cruzi,* showing that they are closely related to the zymodeme Z1 (*T. cruzi* I). Evaluating the potential of each technique as a discriminatory tool: MLEE clearly distinguished two distinct isoenzyme profiles. The genotypic diversity (GD) estimated by the number of multilocus genotypes on the total number of stock was 0.11 (2/18). The GD observed in the *T. cruzi* strains from Jaguaruana was less pronounced than that observed in other sylvatic cycles, for example, in Chile (GD = 0.55) or in Paraguay (GD = 0.64), where 7 different multilocus genotypes were found among 11 stocks (Barnabé *et al*., 2001). A limited multilocus genetic variability similar to that found here, however, was also observed by Thomaz-Soccol *et al*. (2002) in Paraná state, Brazil. Such findings could suggest a founder effect and a recent origin of the *T. cruzi* populations.

The isoenzyme patterns observed in the present study do not, however, appropriately fit in the phenetic tree produced from RAPD profiles. The divergent strain (CE28) does not appear as the most differentiated RAPD genotype in its respective phenetic tree. Nevertheless, results furnished by the technique of RAPD were, otherwise, more informative, suggesting significant intraspecific variation among the *T. cruzi* strains studied. Considering all the RAPD patterns, from the most distinct (CE32) to the very closely related ones, 10 genotypes were found; the GD was therefore 0.55. According to Barnabé *et al*. (2000), when both molecular markers are simultaneously assayed differences are observed probably because isoenzyme data convey less information and more homoplasy than RAPD. Overall, the results of the RAPD-based phenetic analysis provided further support to

the observed intraspecific heterogeneity as it partitioned the isolated strains into distinct clusters. The genotypes were found to be spread through the clusters. The polymorphisms detected in size and in the number of amplified DNA fragments were essential for evaluating the dispersion of the genotypes in the different localities and ecotopes.

Epidemiological Considerations

The localities under study are very poor rural areas of the Jaguaruana municipality in the state of Ceará. The climate is predominantly hot and dry, with a mean annual temperature of 24°C. The vegetation is that of areas of "caatinga" (shrublands), characteristic of the semi-arid northeast region, mainly comprising grassy plants and "carnauba" palm trees. The local economy is typically rural, based on subsistence agriculture (beans, corn, maize, and bananas).

From the epidemiological point of view, it is interesting to draw attention to the nine *T. cruzi* RAPD genotypes found in peridomiciliary ecotopes in the locality of Currais do Felipe. The same *T. cruzi* genotype (CE3 = CE4 = CE8 = CE21 = CE25), belonging to the first phenetic cluster, was found to be circulating in intra- and peridomiciles of three localities (Currais do Felipe, Lagoa Vermelha, and Corrego Salgado). In addition, a dissimilar genotype was found in the locality of Pacatanha. The village of Currais do Felipe, only 7 km from the city center, is separated from the other three localities by the Jaguaribe River. *T. brasiliensis* and *T. pseudomaculata* are native species, prevalent in peridomiciles, and are considered semidomestic Chagas disease vectors (Dias *et al*., 2002). In fact, our group has reported that Currais do Felipe presented the highest index of peridomestic triatomine infection (Sarquis *et al*., 2004). The same report noted continuous deforestation and a gradual reduction of the "carnauba" palm trees for building houses, fences, and corrals. Such annexes (hen houses, and pigsties), normally found in the peridomiciles, are constructed near human dwellings (20 m). Domestic animals such as dogs, cats, sheep, and pigs are frequently found circulating around the houses. All these features are of epidemiological impact and surely contribute to the introduction of *T. cruzi* I lineages into peri- and intradomiciles.

Deforestation is another point that should be taken into account, which leads to the appearance of marsupials and rodents attracted by light and food into human dwellings. Although the two other villages are geographically separated from the aforementioned area by a river (Lagoa Vermelha) and a mountain (Corrego Salgado), the eco-epidemiological characteristics and the anthropic factors could, in part, explain the presence of a widespread genotype such as that found in intra- and peridomiciliary environments of the four localities. On the other hand, the finding of a different genotype in the village of Pacatanha cannot be unequivocally justified from our data, as information regarding eco-epidemiological characteristics is missing. Further genotypic characterizations of *T. cruzi* isolated from wild and

synanthropic mammals from those areas will surely provide new insight into the dynamics of the dispersion of *T. cruzi* I populations into domestic environments. Corroborating such statements, we have recently typed as *T. cruzi* I the isolates from a marsupial (*Didelphis albiventris*) and two synanthropic rodents (*Rattus rattus*) captured in a rural area of Jaguaruana (unpublished results).

The contribution of this paper is, on the one hand, to reinforce the need for constant epidemiological vigilance to prevent the spread of *T. cruzi* I strains into domestic environments. On the other hand, it demonstrates the usefulness of molecular markers in eco-genetic studies.

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