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Multilocus enzyme electrophoresis analysis of *Trypanosoma cruzi* isolates from a geographically restricted endemic area for Chagas' disease in Argentina

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Abstract

A set of 65 *Trypanosoma cruzi* stocks from dogs, opossums, insect vectors and humans was isolated in a geographically restricted endemic area for Chagas' disease in Argentina and was analysed by multilocus enzyme electrophoresis for 15 loci. The results show that at least five multilocus genotypes (clonets) circulate in the study area, one belonging to *T. cruzi* IIe, one to *T. cruzi* IId and three clonets belonging to *T. cruzi* I; and they confirm the presence of these lineages in the country. The three clonets attributed to *T. cruzi* I were identical to each other for all loci except for Sod-2, where three different patterns were identified. These patterns suggest the presence of two homozygous genotypes and one heterozygous genotype. Our results also suggest association of clonet IIe with dogs, clonet IId with humans and the three *T. cruzi* I clonets with *Didelphis albiventris*. On the other hand, there was no significant association between *Triatoma infestans* and any particular clonet circulating in the area. These findings are consistent with the hypothesis of natural selection, from mixed populations of *T. cruzi* in vectors, toward more restricted populations in mammals. The epidemiological implications of the possible selection of different clonets by different mammal hosts and the significance of two homozygous genotypes and one heterozygous genotype for the Sod-2 locus are discussed.

Keywords: *Trypanosoma cruzi*; Multilocus enzyme electrophoresis; Host selection; Hybrid genotype

1. Introduction

Trypanosoma cruzi is the etiological agent of Chagas' disease, which affects several million Latin American people. This parasite exhibits in its natural cycles a high genetic variability (Miles et al., 1978; Morel et al., 1980; Tibayrenc et al., 1985, 1993; Steindel et al., 1993; Souto et al., 1996; Oliveira et al., 1998) and a predominantly clonal population structure (Tibayrenc et al., 1985; Tibayrenc and Ayala, 1988). Natural populations are composed of multiple clones, most of which are distributed into two major phylogenetic lineages, *T. cruzi* I and *T. cruzi* II. The phylogenetic hierarchisation of these main phylogenetic subdivisions is still under debate. Each presents a considerable genetic variability (Tibayrenc, 1995; Souto et al., 1996; Anonymous, 1999). Five lower phylogenetic subdivisions have been identified within *T. cruzi* II lineage, designated *T. cruzi* IIa–e, whereas no clear subdivision was found within *T. cruzi* I (Brisse et al., 2000; Barnabé et al., 2000).

Intraspecific strain characterisation, taking into account our present knowledge on *T. cruzi* phylogenetic structure, including its lesser genetic subdivisions, is crucial in molecular epidemiology of Chagas' disease. Lineage-specific multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD) markers are available for this purpose (Brisse et al., 2000). Below the phylogenetic subdivisions are the natural clones identified by a given set of genetic markers ('clonets') (Tibayrenc and

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Ayala, 1991). The clonets are the lowest level of genetic characterisation in molecular epidemiology. The identification of the clonets circulating in different areas and hosts is important to understand the role of different reservoirs and vectors, to evaluate the possible influence of the infective genotypes in the wide spectrum of clinical outcomes of the disease and to test some biological properties such as sensitivity to antichagasic drugs.

The main surveys concerning the genetic characterisation of *T. cruzi* stocks from Argentina were carried out by Montamat et al. (1987, 1992) and De Luca d'Oro et al. (1993). They identified 12 MLEE genotypes ('zymodemes') from different hosts and regions of Argentina. Later, Barnabé et al. (2000) by analysing published data of De Luca d'Oro et al. (1993) were able to identify 10 of 12 zymodemes from Argentina as belonging to *T. cruzi* I, *T. cruzi* IIb and *T. cruzi* IId lineages.

Stock collections from wide surveys of different localities yield interesting information regarding geographic distribution of lineages. However, the transmission routes may better understood through the analysis of stocks from representative hosts and vectors within a restricted geographical area. No such surveys have so far been carried out in Argentina.

In the present work, we report the results of a genetic survey dealing with MLEE of 65 *T. cruzi* stocks from *Triatoma infestans*, dogs, opossums (*Didelphis albiventris*) and humans from a geographically restricted endemic area for Chagas' disease in Argentina.

2. Materials and methods

2.1. Study area

The field work was carried out in the Department of Chacabuco, Province of Chaco, Argentina. Most of the samples were obtained within an area of 300 km^2, taking as central point the settlement of Tres Estacas (26° 55′11″S; 61° 37′42″W), from January 1999 to November 2001. According to its biogeographical characteristics, the area belongs to the Chaqueña Region (Cabrera and Willink, 1973) and exhibits patches of primary and secondary forest alternated with crop fields and dispersed human dwellings. The National Control Agency sprayed this area with deltamethrin in 1996. After fumigation, reinfestation by *T. infestans* was detected. At the time of sampling, the seroprevalence in children was 28.67% and many houses showed intra-domiciliary *T. infestans* infected by *T. cruzi* (Diosque et al., 2000).

2.2. Parasite isolation and reference strains

Sixty-five stocks of *T. cruzi* were isolated, 38 from *T. infestans*, 16 from dogs, seven from *D. albiventris* and four from humans. Stocks from dogs and opossums were collected by xenodiagnosis using 30 fourth instar nymphs of *T. infestans*. Bugs captured inside houses and in the peridomiciliary environment, as well as nymphs used for xenodiagnosis were examined by fresh faeces smears. The positive samples were inoculated in BALB/c mice and later these mice were submitted to hemoculture in liver infusion tryptose (LIT) medium. Human isolates were obtained by hemoculture in LIT medium. The isolated parasites were maintained by passages in LIT medium at 28 °C. Four strains were used as reference: X10cl1 (*T. cruzi* I), CANIIIcl1 (*T. cruzi* IIa), Mncl2 (*T. cruzi* IId) and CL-Brener (*T. cruzi* IIe). X10cl1 and CANIIIcl1 correspond to the formerly described zymodemes I and III, respectively (Miles et al., 1978).

2.3. Sample preparation

Stocks were harvested by centrifugation (2800 × *g*, 20 min, 4 °C) and washed in PBS (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.2). Cells were lysed on ice for 20 min in an equal volume of hypotonic enzyme stabiliser (EDTA 2 mM, dithiothreitol 2 mM, ε-aminocaproic acid 2 mM). The soluble fraction was stored at − 70 °C until used in MLEE analysis.

2.4. Isoenzyme analysis

Electrophoresis on cellulose acetate plates was performed as described by Ben Abderrazak et al. (1993) with slight modifications. Twelve enzyme systems were assayed, namely, diaphorase (EC 1.6.99.2, DIA), glutamate dehydrogenase NAD + (EC 1.4.1.2, GDH-NAD +), glutamate dehydrogenase NADP + (EC 1.4.1.4, GDH-NADP +), glutamate oxaloacetate transaminase (EC 2.6.1.1, GOT), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD), glucose-6-phosphate isomerase (EC 5.3.1.9, GPI), isocitrate dehydrogenase (EC 1.1.1.42, IDH), malate dehydrogenase (EC 1.1.1.37, MDH), malic enzyme (EC 1.1.1.40, ME), peptidase 1 (EC 3.4.22.3, PEP-1; substrate leucyl-leucyl-leucine), phosphoglucomutase (EC 5.4.2.2, PGM) and superoxide dismutase (EC 1.15.1.1, SOD).

2.5. Data analysis

MLEE patterns were compared with those described for the major lineages and for additional subdivisions of *T. cruzi* II lineage (Brisse et al., 2000; Barnabé et al., 2000) using the four reference strains. Every band was counted, excepting central bands in heterozygous patterns that showed three bands. These patterns in *T. cruzi* have been attributed to heterozygous genotypes for dimeric enzymes, in which central bands do not correspond to an allele (Tibayrenc et al., 1985). Genetic and genotype diversity was estimated with various indices, and population structure of the sample was explored by tests of linkage disequilibrium. All indices and

Table 1
Genetic variability indices and linkage disequilibrium tests

Genetic variability indices
Polymorphism rate: $P = n_i/n$ where (n_i) is the number of polymorphic loci and (n) is the total number of loci, considering as monomorphic a locus for which more than either 95% or 99% of the individuals share the same genotype, according to the threshold selected
Mean genetic diversity: $H = \sum hij/n$ where (n) is the number of loci and $(hij) = 1 - \sum xij^2$ where (xij) is the relative frequency of the ith electromorph at the jth locus
Clonal diversity: $D = n(1 - \sum xi^2)/n - 1$ where (n) is the number of individuals and (xi) is the relative frequency of the ith multilocus genotype
Genotypic diversity: $G = g/n$ where (n) is the number of individuals examined and (g) the number of different multilocus genotypes

Linkage disequilibrium tests (Tibayrenc et al., 1991)
d1 = combinatorial probability of sampling the most common multilocus genotype as often or more than actually observed, given by the formula: $P = \sum_{i=m}^{n}(n!\cdot x^i\cdot(1-x)^{n-i}/i!\cdot(n-i)!)$, where ($x$) is the expected probability of the multilocus genotype, (n) is the number of individuals in the sample, and $i = m$ is the number of individuals showing this particular genotype
d2 = probability of observing any multilocus genotype as often as or more often than the most common multilocus genotype in the sample[a]
e = probability of observing as few or fewer multilocus genotypes in the population than observed in the sample[a]
f = probability of observing a linkage disequilibrium in the population as high or higher than observed in the sample[a]

[a] Performed by a Monte Carlo simulation using 10^4 iterations.

tests are listed in Table 1. The chi-square test was used to analyse the host distribution of the clonets.

3. Results

3.1. Multilocus enzyme electrophoresis analysis

The DIA, ME and SOD enzyme systems exhibited two zones of activity, which are believed to be determined by two separate gene loci (*Dia*-1, *Me*-1 and *Sod*-1 for the fastest migrating enzymes, and *Dia*-2, *Me*-2, *Sod*-2 for the slowest ones). This gives a total of 15 interpretable genetic loci.

Five multilocus genotypes or clonets were detected out of the 65 analysed stocks. One clonet pertained to *T. cruzi* IId lineage, according to the specific character *Gpi 2/4* genotype, and appeared identical to the Mncl2 reference strain. The second clonet belonged to *T. cruzi* IIe (*Gpi 3/4* genotype) and was identical to CL-Brener reference strain. The three remaining clonets were attributed to *T. cruzi* I (*Gpi 5/5* genotype). These three clonets were identical to each other for all loci except for *Sod*-2, where three different patterns were identified. One pattern exhibited a unique slow migrating band (*Sod-2 2/2* genotype). The second pattern showed a unique fast migrating band (*Sod-2 1/1* genotype). The last pattern was three-banded, compatible with heterozygous genotype for a dimeric enzyme (*Sod-2 1/2* genotype) (Fig. 1). The three clonets of the *T. cruzi* I lineage identified in this study were different from the X10cl1 reference strain at the *Pep*-1 locus. Table 2 gives

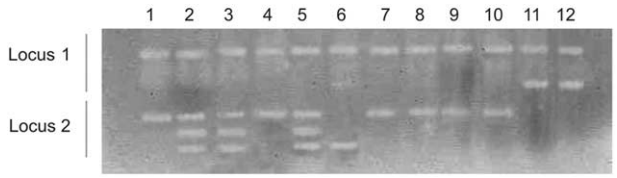

Fig. 1. Patterns for superoxide dismutase (SOD). Lines 1–10, *Trypanosoma cruzi* I. Lines 1, 4, 7, 8, 9 and 10, genotype 1/1; lines 2, 3 and 5, genotype 1/2 and line 6, genotype 2/2 for *Sod*-2 locus. Line 4, X10cl1 (reference strain for *T. cruzi* I); line 11, Cl-Brener (reference strain for *T. cruzi* IIe); line 12, isolate belonging to *T. cruzi* IIe.

the genotypes of the 15 loci under study for the five clonets identified and the four reference strains. Fig. 2 shows the *Gpi* diagnostic genotypes for the three lineages identified in this work, including the reference strains.

3.2. Genetic variability indices, genotype diversity and population structure

Results of variability indices and results of linkage disequilibrium tests are summarised in Table 3. Linkage disequilibrium was recorded in every test for the whole sample and for *T. cruzi* II, except test e for *T. cruzi* II.

3.3. Phenotype frequencies for the Sod-2 locus

Table 4 shows the frequencies of *Sod-2* genotypes for the stocks belonging to *T. cruzi* I. No significant differences were found between expected and observed frequencies.

3.4. Host distribution of the clonets

The observed distribution of the clonets among the different hosts is significantly different from the expectations of a random distribution. *Trypanosoma cruzi* I was over-represented in *Didelphis albiventris* (7/7; $P = 9$ E-6). *Trypanosoma cruzi* IIe was over-represented in dogs (14/16; $P = 3.7$ E-4) and four of four human isolates were *T. cruzi* IId ($P = 2.4$ E-4). All these five clonets were found in *T. infestans* without any specific association with this vector. Table 5 shows the distribution of the five clonets in the different hosts.

4. Discussion

4.1. Host specificity of the clonets

Previous isoenzymic studies of *T. cruzi* in Argentina were carried out by analysing isolates from different regions of the country (Montamat et al., 1987, 1992; De Luca d'Oro et al., 1993) or from wild mammals from a restricted area (Wisnivesky-Colli et al., 1992). This is the first isoenzymic study of *T. cruzi* covering representative collections of hosts and vectors from a restricted geographic area in Argentina.

Table 2
Multilocus genotypes of the five clonets and the four reference strains of *Trypanosoma cruzi* characterised with 15 loci[a]

	GPI	GOT	PGM	IDH	MDH	PEP1	G6PD	GDH1	GDH2	ME1	ME2	DIA1	DIA2	SOD1	SOD2
T. cruzi I′[b]	5/5	3	1/1	3	3/3	1/1	2	2	1/1	1/1	2/2	2/2	1/1	1-1	1/1
T. cruzi I″[b]	5/5	3	1/1	3	3/3	1/1	2	2	1/1	1/1	2/2	2/2	1/1	1-1	1/2
T. cruzi I‴[b]	5/5	3	1/1	3	3/3	1/1	2	2	1/1	1/1	2/2	2/2	1/1	1-1	2/2
T. cruzi IId	2/4	2	2/4	5	3/3	2/2	1	1	1/1	1/1	3/3	3/3	1/1	1-2	0/0
T. cruzi IIe	3/4	2	2/4	5	3/3	2/2	1	1	1/1	1/1	3/3	3/3	1/2	1-2	0/0
X10cl1 (Lineage I)[c]	5/5	3	1/1	3	3/3	2/2	2	2	1/1	1/1	2/2	2/2	1/1	1-1	1/1
Mncl2 (Lineage IId)[c]	2/4	2	2/4	5	3/3	2/2	1	1	1/1	1/1	3/3	3/3	1/1	1-2	0/0
CL-Brener (Lineage IIe)[c]	3/4	2	2/4	5	3/3	2/2	1	1	1/1	1/1	3/3	3/3	1/2	1-2	0/0
CANIIIcl1 (Lineage IIa)[c]	4/4	2	3/3	5	3/3	3/3	3	1	2/2	1/1	1/1	2/2	2/2	1-2	0/0

[a] An allelic interpretation of the enzymatic profiles was made for all loci except GOT, IDH, DIA1 and SOD1.
[b] In this table *T. cruzi* I′, I″ and I‴ are exclusively used to name the three different clonets belonging to *T. cruzi* I, and do not represent lineages formally defined.
[c] Reference strain.

The results of the present work show that at least five clonets circulate in the study area, one belonging to *T. cruzi* IId, one to *T. cruzi* IIe and three clonets belonging to *T. cruzi* I; and confirm the presence of these lineages in the country. Our results suggest association of clonet IIe with dogs, clonet IId with humans and *T. cruzi* I with *D. albiventris*. On the other hand, there was no significant association between *T. infestans* and any particular clonet circulating in the area. These findings are consistent with the hypothesis of natural selection, from mixed populations of *T. cruzi* in vectors, toward more restricted populations in mammals.

The association of clonet IIe with dogs is supported by 14/16 dogs infected by this clonet ($P = 3.7$ E-4). The association between clonet IId and humans is statistically significant ($P = 2.4$ E-4), but it is supported by just four isolates. More samples will be necessary to test this hypothesis.

Our results also suggest the existence of a sylvatic cycle in the study area. The presence of *T. cruzi* I parasites in *D. albiventris*, which were captured in nearby forests, agrees with both, previous work indicating that *Didelphis sp*. is the primary reservoir of the parasite in America (reviewed by Barreto, 1979) and the high frequencies of this lineage in wild cycles (Barnabé et al., 2000). Although just one dog, two peridomestic and five domestic *T. infestans* were infected with *T. cruzi* I parasites, the presence of this lineage in domestic environment suggests that domestic and wild cycles are partially overlapped in the study area.

We cannot rule out possible selection of different clonets during the isolation process. However, excepting the human isolates (derived directly by hemoculture in LIT medium) every isolate was obtained in this work by the same procedure. In spite of this, we were able to isolate the five different clonets identified in this study. Moreover, the possibility of selection by isolation procedures is not compatible with the finding of five clonets being present in *T. infestans* without significant association of any particular clonet with this vector. Some observations during the culture routine led us to think that *T. cruzi* I clonets grow up faster than *T. cruzi* II clonets, and that IIe clonets grow up faster than IId. If this was true, we would have been unable to detect mixed infections in some cases, because of the loss of some clonets during the isolation process.

Effective control and even elimination of infection with different clonets of *T. cruzi* by the immune system of different mammals has been experimentally proved (Deane et al., 1984; Jansen et al., 1999). This has also been suggested by field data (Breniere et al., 1998). Bosseno et al. (1996) found the two principal clonets infecting domestic *T. infestans* in Bolivia (clonet 20 belonging to *T. cruzi* I, and clonet 39 belonging to *T. cruzi* IId) in similar frequencies and often associated in a single vector. Later, Breniere et al. (1998), by analysing patients of the same area, and using the polymerase chain reaction and clonet-specific kinetoplast DNA probes, found almost exclusively clonet 39 (IId) in these patients. They suggested a drastic control by the immune system of patients and a possible role of the synanthropic mammals in the maintenance of the clonet 20 (*T. cruzi* I). Such an epidemiological model is in agreement with our results, suggesting that *T. cruzi* IId is principally maintained by humans and *T. cruzi* IIe by dogs. Parasites belonging to *T. cruzi* I would be most probably maintained

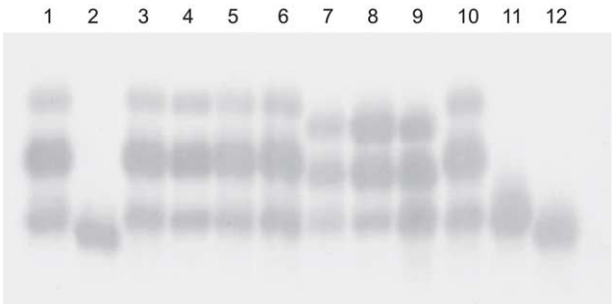

Fig. 2. Patterns for glucose phosphate isomerase (GPI). Line 2, X10cl1 (reference strain for *Trypanosoma cruzi* I); line 4, Mncl2 (reference strain for *T. cruzi* IId); line 9, CL-Brener (reference strain for T. *cruzi* IIe); line 11, CANIIIcl1 (reference strain for *T. cruzi* IIa). On the remaining lines isolates characterised in this study. Lines 1, 3, 5, 6 and 10, *T. cruzi* IId; lines 7 and 8, *T. cruzi* IIe; line 12, *T. cruzi* I.

Table 3
Genetic variability indices and levels of significance of the linkage disequilibrium tests for *Trypanosoma cruzi*

	NS	ND	P99[a]	MGD	CDI	GD	d1	d2	e	f
Whole sample	65	5	0.80	0.31	0.68	0.076	0.00	<10e-4	<10e-4	<10e-4
T. cruzi I	15	3	0.07	0.03	0.49	0.200	0.62	1	1	1
T. cruzi II	50	2	0.13	0.06	0.50	0.040	2.7e-04	<10e-4	1	<10e-4
T. cruzi IId	22	1	0.00	0.00	0.00	0.045	–	–	–	–
T. cruzi IIe	28	1	0.00	0.00	0.00	0.036	–	–	–	–

NS, number of stocks; ND, number of different multilocus genotypes; P99, polymorphism at 99%; MGD, mean genetic diversity; CDI, clonal diversity index; GD, genotypic diversity; d1, d2, e and f, linkage disequilibrium tests, see Table 1.
[a] Values of polymorphism at 99% were equal to values at 95%.

by synanthropic mammals different from dogs or introduced from the wild cycle to domestic environment in our study area.

The possibility that chagasic infection is due principally to *T. cruzi* II parasite, has been suggested by biological and epidemiological data, and more recently by results of DiNoia et al. (2002). They showed, by using an immunological marker able to differentiate *T. cruzi* I and *T. cruzi* II infection, that more than 87% of chagasic patients' sera from Argentina, Chile and Brazil were reactive for the antigen specific for *T. cruzi* II, whereas only 5% of these sera displayed a mixed recognition (*T. cruzi I* and *T. cruzi* II co-infections). None of chagasic patients analysed showed an exclusive *T. cruzi* I infection. These data suggest that, at least in the Southern Cone's countries of Latin America, the human infection by *T. cruzi* are due principally to *T. cruzi* II (or even a subgroup of this lineage).

The possible selection of different clonets by different mammal hosts could have important epidemiological implications. Cohen and Gürtler (2001), by using a mathematical model calibrated to detailed household data, proposed a very important role of infected dogs in the risk of human infection. If selection of different clonets by different mammal hosts occurs, such as our results suggest, the role of different host in domestic transmission of *T. cruzi* could be highly dependent on the clonets circulating in each area.

4.2. Population structure and genetic exchange

When the *Sod-2* locus is considered, the patterns observed suggest the presence of two homozygous genotypes and one heterozygous genotype. The bands of the two homozygous genotypes match with the fastest and the slowest bands of the heterozygous genotype.

The finding of two different homozygous genotypes and the corresponding heterozygous genotype for the *Sod-2* locus in *T. cruzi* I clonets in the present work is interesting in view of some results obtained by other investigators. Carrasco et al. (1996) made a similar finding for the phosphoglucomutase locus studying isolates belonging to *T. cruzi* I from a wild cycle in Brazil. They found two different homozygous and the corresponding heterozygous genotype with an observed frequency almost identical with that predicted by the theoretical Hardy–Weinberg distribution. Parental and hybrid profiles were also suggested by RAPD analysis. There are other studies that report genetic exchange obtained experimentally in *T. cruzi* (Stothard et al., 1999) and presence of heterozygotes and homozygotes among clinical isolates (Bogliolo et al., 1996). Moreover, Machado and Ayala (2001), by analysing a large data set of nucleotide sequences from two nuclear genes and a region of the mitochondrial genome, showed evidence of hybridisation between strains from two divergent groups of *T. cruzi*, and revealed genetic exchange among closely related strains.

Table 4
Frequencies of superoxide dismutase-2 (*Sod*-2) genotypes for 15 isolates of *Trypanosoma cruzi* I[a]

Genotype (no. of isolates)	Frequency	
	Observed	Expected[b]
1/1 (4)	0.27	0.36
1/2 (10)	0.67	0.48
2/2 (1)	0.06	0.16

[a] None of the differences between observed and expected distribution are significant by χ^2 test ($P > 1$).
[b] For a population in Hardy–Weinberg equilibrium.

Table 5
Host distribution of the clonets of *Trypanosoma cruzi*

Clonets	Host				Total
	Didelphis albiventris	*Triatoma infestans*	Human	Dog	
T. cruzi I (*Sod*-2 1/1)	0	3	0	1	4
T. cruzi I (*Sod*-2 1/2)	6	4	0	0	10
T. cruzi I (*Sod*-2 2/2)	1	0	0	0	1
T. cruzi IId	0	17	4	1	22
T. cruzi IIe	0	14	0	14	28
Total	7	38	4	16	65

The presence of hybrid genotypes in *T. cruzi* has been also proposed by Brisse et al. (2003). The possible mechanism involved in these genetic exchanges is yet unclear, but recent experimental data suggest that fusion of parental genotypes, loss of alleles, homologous recombination and uniparental inheritance of kinetoplast maxicircle DNA do occur (Gaunt et al., 2003). However, all these data do not reject the hypothesis of a basically clonal population structure in *T. cruzi* (Tibayrenc et al., 1985; Tibayrenc and Ayala, 1988), but they suggest that genetic exchange could contribute to the generation of genetic diversity in this parasite. In our sample (15 isolates of *T. cruzi* I) the frequencies of the two different homozygous and the heterozygous genotypes for *Sod-2* are not significantly different from that predicted by the theoretical Hardy–Weinberg distribution. However, the very limited size of this sample generates a high risk of statistical type II error (impossibility to reject the null hypothesis of Hardy–Weinberg equilibrium by lack of power of the test). It is hardly acceptable that this population undergoes random genetic exchange, since almost all linkage disequilibrium tests are highly significant. This shows that genetic recombination is severely limited in this *T. cruzi* population, which therefore exhibits the same prevalent clonal evolution picture than other *T. cruzi* populations. The lack of significance of the linkage disequilibrium tests within the isolates attributed to *T. cruzi* I in the present study can be explained by a statistical type II error, since both sample size and genetic variability are quite limited. Of course, we cannot rule out a possible role played by genetic exchange. The most relevant fact is that we found a putative hybrid genotype in *T. cruzi* lineage I, whereas most other studies reporting the existence of such hybrid genotypes mainly concerned *T. cruzi* lineage II.

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