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Trypanosoma cruzi: Compared Vectorial Transmissibility of Three Major Clonal Genotypes by *Triatoma infestans*

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de Lana, M., da Silveira Pinto, A., Barnabé, C., Quesney, V., Noël, S., and Tibayrenc, M. 1998. *Trypanosoma cruzi*: Compared vectorial transmissibility of three major clonal genotypes by *Triatoma infestans*. *Experimental Parasitology* **90**, 20–25. Twenty *Trypanosoma cruzi* stocks attributed to the 19/20, 32, and 39 major clones (Tibayrenc *et al.* 1986) were used to infect experimentally third instar larvae of *Triatoma infestans*. Three variables were considered: (i) percentage of infected insects; (ii) number of flagellates per insect (NFI); and (iii) percentage of metacyclic trypomastigotes per insect. Differences between the genotypes under study for all parameters considered were detected. These differences were statistically significant ($P < 10^{-3}$), except between the 39 and 32 clonal genotypes for the NFI parameter. The correlation coefficient between the genetic distance and the biological parameters determined by the nonparametric Mantel's test was strongly significant ($P < 10^{-4}$). Data obtained suggest clearly that populations of parasites belonging to the 19/20 genotype are more efficiently transmitted (high transmissibility genotype) by the vector than the 32 genotype (low transmissibility genotype), while the 39 genotype presents intermediary characteristic. Results confirm the working hypothesis that the subdivision of *T. cruzi* into discrete clonal lineages has an impact on the vectorial competence of *T. infestans*, the most important vector of the chagasic infection in South America, and that different clonal lineages do not exhibit the same vectorial transmissibility. This fact is relevant both for Chagas' disease epidemiology and for the use of xenodiagnosis.

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Index Descriptors and Abbreviations: Trypanosoma cruzi; phylogenetic divergence; clonal structure; biological variability; multilocus enzyme electrophoresis (MLEE); random amplification of polymorphic DNA (RAPD); percentage of infected insects (%II); number of flagellates per insect (NFI); percentage of metacyclic trypomastigotes per insect (%DIF).

INTRODUCTION

Chagas' disease remains a major public health problem in Latin America. Its causative agent, the protozoan *Trypanosoma cruzi*, undergoes a complex life cycle passing through both triatomine bugs and mammals. Within the gut of the vector, the ingested bloodstream trypomastigotes undergo differentiation into proliferative epimastigotes that become infective metacyclic trypomastigotes in the rectum (Brener 1973). The relationships between the parasite's diversity and its vectorial transmissibility are not well known.

Studies have shown that *T. cruzi* populations differ in their ability to survive, multiply, and differentiate in the insect vector (Urdaneta-Morales and Rueda 1977; Garcia and Dvorack 1982; Schaub 1989; Mello *et al.* 1996).

Apart from vectorial transmissibility, the diversity of *T. cruzi* populations has been explored by general biological behavior (Andrade 1976), growth kinetic (Dvorak *et al.*

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1980), isoenzyme variability (Miles *et al.* 1980; Andrade *et al.* 1983; Tibayrenc and Ayala 1988), pathogenic properties (Miles *et al.* 1981), kinetoplast DNA restriction fragment polymorphism (Morel *et al.* 1980), and random amplified polymorphic DNA (RAPD) (Steindel *et al.* 1993; Tibayrenc *et al.* 1993).

Population genetic analyses have shown that *T. cruzi* has a typical clonal population structure (Tibayrenc *et al.* 1986; Tibayrenc and Ayala 1988). Among the natural clones evidenced by genetic markers, some are widespread and much more frequently sampled than others. They have been named: "major clones" (Tibayrenc and Ayala 1988; Tibayrenc and Breniere 1988). Moreover, it has been shown that clonal variability is statistically linked to relevant biological parameters such as culture growth, virulence in mice, and *in vitro* drug sensitivity (Laurent *et al.* 1997; Revollo *et al.* in press).

The present work aims at exploring the hypothesis that *T. cruzi* clonal diversity has an impact on its transmissibility through insect vectors. More specifically, as for previous experimental studies ((Laurent *et al.* 1997; Revollo et al. in press), the working hypothesis proposed here is that *T. cruzi* biological diversity (here: vectorial transmissibility) is statistically correlated to the phylogenetic divergence that is observed among the parasite's natural clones.

MATERIALS AND METHODS

(1) Parasites. The same standardized sample used for previous experimental studies (Laurent *et al.* 1997; Revollo *et al.* in press) has been used. All stocks have been fully characterized by both MLEE with 22 different genetic loci and RAPD (Tibayrenc *et al.* 1993). They have been cloned in the laboratory, with verification under the microscope. Information on the laboratory code, host, and geographic origin of the stocks under survey is given in Table I. This set of 20 stocks is representative of three major clonal lineages of *T. cruzi*, numbered 19/20, 32, and 39, according to the coding by Tibayrenc *et al.* (1986). Major clones 19, 20, 32, and 39 characterized by 15 enzyme loci (Tibayrenc *et al.* 1986) showed limited additional variability with the use of more discriminative markers (Tibayrenc *et al.* 1993), as expected. They should be considered as families of closely related clones rather than actual clones (Tibayrenc and Ayala 1988). Moreover, the 19/20 group, formerly distinguished into 19 and 20 natural clones on the basis of 15 isoenzyme loci (Tibayrenc *et al.* 1986), is less clearly separated with more discriminative methods (Tibayrenc *et al.* 1993). In the present study, they are plotted together into a unique group of closely related clonal genotypes, designated 19/20. The three categories of clonal genotypes, 19/20, 32, and 39, illustrate different phylogenetic relationships: 32 and 39 are more closely related to each other, while 19/20 is more distantly related to both 32 and 39 (see Fig. 1).

(2) Experimental conditions. Experiments were undertaken with third instar nymphs of *T. infestans* reared in laboratory conditions, i.e., at about 26–27°C, 65–70% relative humidity, and allowed to feed on chickens every 3 weeks. The insects originate from an outbred colony with individuals coming from Chile, Uruguay, and Brazil. The insects were exposed to infection by using middle-log phase cultures forms from LIT medium, at 28°C in an artificial xenodiagnosis device through latex membranes. Eight milliliters of parasite suspension in citrated mice blood, at the final concentration of 5.0×10^5 cells/ml, was used. The system was maintained at 37°C and continuously homogenized with a magnetic stir bar. Only engorged insects were considered.

For parasite quantification, 30 days later, the whole digestive tube was removed and gently ground in 0.6-ml Eppendorf tubes containing 10 μl of phosphate-buffered saline (PBS, pH 7.2). The suspension was then homogenized and used to prepare fresh slide smears covered with 22×22 lids. Preparations were scored microscopically for the presence of flagellates, total number of flagellates, and percentage of metacyclic trypomastigotes. Two hundred fields for each preparation were examined. Additionally, the entire intestinal tract of two insects for each stock was aseptically removed and introduced into 15-ml Falcon tubes containing 3.0 ml of LIT medium with 50 μg/ml of gentamicin. The tubes were kept at 28°C and weekly examined for the presence of flagellates. The clonal genotype of these isolates was verified by isoenzymes. Cellulose acetate electrophoresis was performed under conditions previously described (Ben Abderrazak *et al.* 1993). Five enzyme systems were assayed: phosphoglucose isomerase (EC 5.3.1.9, PGI); phosphoglucomutase (EC 5.4.2.2, PGM); isocitrate dehydrogenase (EC 1.1.1.42, IDH); 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6PGD); and glutamate oxalate transaminase (EC 2.6.1.1, GOT). This was sufficient to distinguish among the three groups of clonal genotypes surveyed here.

(3) Data analysis. For each stock, three variables were considered: (i) the percentage of infected insects (%II); (ii) the number of flagellates per insect (NFI), and the percentage of metacyclic trypomastigotes per insect (%DIF). These variables were estimated on the 20 stocks representative of the 19/20, 32, and 39 clonal genotypes of *T. cruzi* (see Table I). At least 30 insects were exposed to each stock. A total of 620 engorged insects was considered. The reduced deviation test (SAS Program, version 5-06) was used to compare the parameters between the three categories of clonal genotypes (19/20, 32, and 39). Comparisons of percentages were used in the case of the %II parameter, while comparisons of means were used in the case of the two other parameters.

Moreover, for each possible pair of stocks (190 pairwise comparisons), the absolute difference for the quantified result of each biological parameter was evaluated. An overall "biological distance" was calculated as follows. For each biological parameter, the highest value of absolute difference in all pairs of stocks was 1. The lowest value was 0. The other values were expressed in percentages of the highest value. For each pair of stocks, the overall "biological distance" was given by the arithmetic means of all the values obtained according to this procedure for the three biological parameters under study. This procedure gives an equal weight to each biological parameter in the overall biological distance. Correlations between this overall biological distance, on one hand, and genetic distances measured from either MLEE or RAPD analysis, on the other hand, were then evaluated with a nonparametric Mantel test (Mantel 1967). Briefly, this test is based on a Monte Carlo simulation with 10^4 iterations, which randomly permutes

TABLE I
List of the 20 *Trypanosoma cruzi* Stocks Under Study

Stock	Country	Place	Host
SP104cl1 (19/20)	Chile	IV Region	*Triatoma spinolai*
Cutia cl4 (19/20)	Brazil	Espirito Santo	*Dasylprocta agudi*
Gamba cl1 (19/20)	Brazil	Sao Paulo	*Didelphis azarae*
13379 cl7 (19/20)	Bolivia	Santa Cruz	Man, acute form
OPS21 cl11 (19/20)	Venezuela	Cojedes	Man
SO34 cl4 (19/20)	Bolivia	Toropalka (Potosi)	*Triatoma infestans*
Cuica cl1 (19/20)	Brazil	Sao Paulo	*Opossum cuica philander*
P/209 cl1 (19/20)	Bolivia	Sucre	Man, chronic form
Esquilo cl1 (19/20)	Brazil	Sao Paulo	*Sciurus aestuans ingramini*
P/11 cl2 (19/20)	Bolivia	Cochabamba	Man, chronic form
SC43 cl1 (39)	Bolivia	Santa Cruz	*Triatoma infestans*
Bug2148 cl1 (39)	Brazil	Rio Grande do Sul	*Triatoma infestans*
Bug2149 cl1 (39)	Brazil	Rio Grande do Sul	*Triatoma infestans*
SO3cl5 (39)	Bolivia	Otavi (potosi)	*Triatoma infestans*
MN cl2 (39)	Chile	IV Region	Man, chronic form
MAS1 cl1 (32)	Brazil	Brasilja	Man
CBB cl3 (32)	Chile	Tulahen	Man
Tu18 cl2 (32)	Bolivia	Tupiza	*Triatoma infestans*
IVV cl4 (32)	Chile	Santiago	Man, chronic form
MVB cl8 (32)	Chile	Santiago	Man, chronic form

Note. Clonet number, (in parentheses) refers to the major clonal genotypes previously identified by multilocus enzyme electrophoresis with 15 genetic loci (Tibayrenc and Ayala 1988).

the different cells of one of the distance matrices. Different from the classical correlation test, this randomization procedure does not need any assumptions about the number of degrees of freedom.

RESULTS

In the experimental conditions used, it was observed intuitively that the three different clonal genotypes of *T. cruzi* surveyed here tend to behave differently in *T. infestans*, i.e., stocks belonging to distinct genotypes of the parasite differ in their ability to complete the life cycle in the digestive tract of the insect vector. This was fully confirmed by statistical analysis, although the standard deviations of the values recorded were high (see Table II). All comparison tests between the three different clonal genotypes based on the reduced deviation statistics were highly significant ($P < 10^{-3}$), except in the case of the comparison between the stocks attributed to the 32 and 39 genotypes for the NFI parameter, which showed no significant differences.

The general tendency was that the group 19/20 showed the highest values, the group 32 showed the weakest values, and the 39 group showed intermediary values.

The correlation coefficient between the genetic distances and the biological differences estimated by the Mantel test was also strongly significant ($<10^{-4}$). This shows that those stocks that are genetically related to each other tend to behave similarly in *T. infestans*, which is contrary to those stocks that are genetically distantly related.

Lastly, the isoenzyme profiles of all the stocks under survey showed no change after passage through the insect vector (data not shown).

DISCUSSION

The set of stocks under survey represents a convenient sample for testing the hypothesis of linkage between phylogenetic diversity of *T. cruzi* natural clones and a relevant biological property of the parasite, namely its transmissibility through a major vector species, *T. infestans*. The results show the same overall pattern from previous studies of our group dealing with the same sample of stocks and other biological parameters (Laurent *et al.* 1997; Revollo *et al.* in press). First, within each genotype category taken separately,

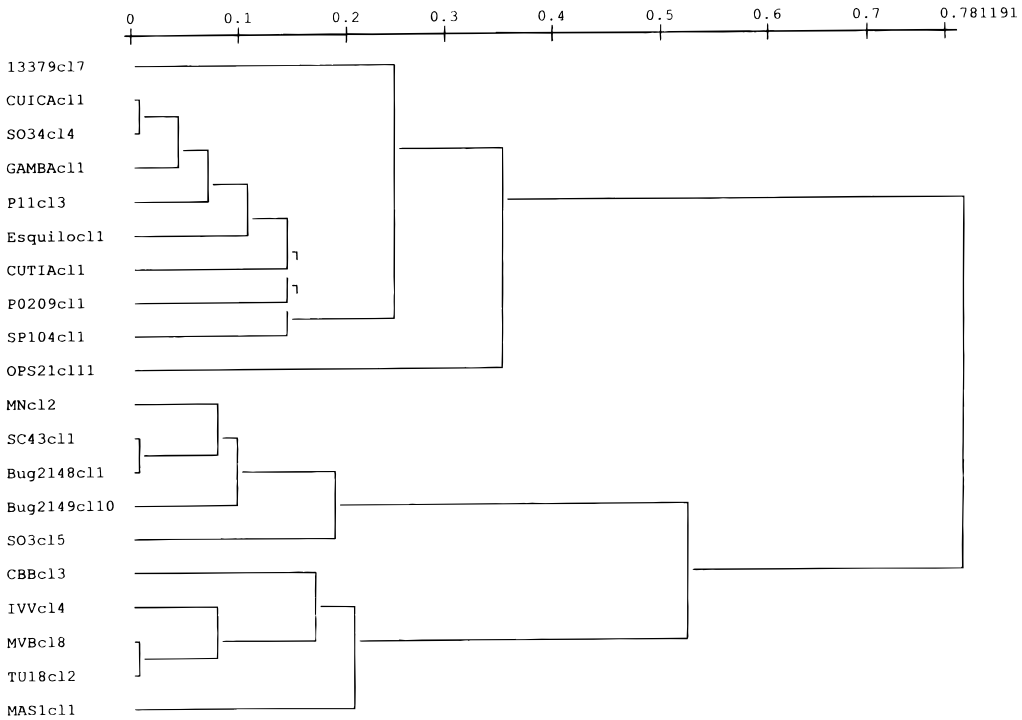

FIG. 1. An UPGMA (unweighted pair-group method with arithmetic averages) dendrogramme (Sneath and Sokal 1973) depicting the phylogenetic relationships among the 20 *Trypanosoma cruzi* stocks under study, assayed by 22 isoenzyme loci (Tibayrenc *et al.* 1993). Top cluster corresponds to the clonal genotype 19/20, medium cluster corresponds to the clonal genotype 39, and bottom cluster corresponds to the clonal genotype 32 (genotype numbering after Tibayrenc and Ayala 1988). The scale indicates genetic distances estimated with the index of Jaccard (1908).

there is a notable variability of the biological parameters, as evidenced by the strong values of standard deviation for the NFI and %DIF parameters (see Table II). This means that the stocks pertaining to a given clonal genotype are not homogeneous for these biological parameters. An additional, possible explanation for this result is that the insect vectors used are themselves genetically heterogeneous, which could interfere with their vectorial competence. Second, despite these notable standard deviations, there is a strong statistical correlation between phylogenetic divergence among *T. cruzi* natural clones on one hand and biological differences (here: transmissibility through *T. infestans*) on the other hand. This is confirmed by both (i) pairwise comparison by the reduced deviation test of clonal genotypes for each parameter taken separately, and (ii) overall correlation evaluated by the Mantel test between genetic distances (phylogenetic divergences) on one hand and overall biological differences on the other hand. Especially remarkable is the different ability of the three clonal genotypes to differentiate into infective metacyclic trypomastigotes, a property that conditions potentially their ability to infect mammalian hosts including humans.

TABLE II

Average Values of the Three Biological Parameters Measured for the Transmissibility of the 20 *Trypanosoma cruzi* Stocks (see Table I) through *Triatoma infestans*

Clonal genotype	%II	NFI	%DIF
19/20	81.76	103,716 ± 201,048	10.48 ± 16.60
39	68.12	51,144 ± 138,132	5.84 ± 11.95
32	51.33	31,380 ± 91,560	0.51 ± 2.27

Note. %II, percentage of infected insects; NFI number of flagellates per insect; %DIF percentage of metacyclic trypomastigotes per insect.

This value was high for the 19/20 clonal genotype, and very weak for the 32 genotype, while the 39 genotype showed intermediary values (see Table II). More generally, this study confirms a notable tendency of previous ones (Laurent *et al.* 1997; Revollo *et al.* in press). By comparison with stocks attributed to clonal genotypes 32 and 39, stocks attributed to clonal genotype 19/20 (i) grow more quickly in *in vitro* culture; (ii) differentiate more easily in culture cells;

(iii) infect more easily mice, and are more virulent for them; (iv) are less sensitive *in vitro* to Rochagan and Nifurtimox; (v) show higher transmissibiliy through *T. infestans* (the present study).

In the case of the present study, it is obvious that different transmissibility and different ability to differentiate into infective trypomastigote forms have a potential impact on both transmission cycles and the routine use of xenodiagnosis. Since the repartition of *T. cruzi* clonal genotypes differs among endemic countries (Tibayrenc and Ayala 1988), the transmission patterns could be modified by the clonal genotypes present in given areas, as well as the sensitivity of the xenodiagnosis.

Our work confirms that it is misleading to consider *T. cruzi* as a homogeneous entity and that phylogenetic divergence among *T. cruzi* natural clones is a parameter that deserves to be taken into account for all applied studies dealing with this parasite.

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