

Isoenzyme Profiles of *Trypanosoma cruzi* Stocks from Different Areas of Paraguay

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Twenty one Trypanosoma cruzi stocks from humans, domiciliary triatomines and one sylvatic animal of different areas of Paraguay were subjected to isoenzyme analysis. Thirteen enzyme systems (15 loci in total) were studied. MN cl2 (clonets 39) and SO34 cl4 (clonets 20) were used as references. Relationships between stocks were depicted by an UPGMA dendrogram constructed using the Jaccard's distances matrix. Among the Paraguayan stocks 14 zymodemes were identified (Par1 to Par14), Par 5 being the most frequent. Polymorphism rate and clonal diversity were 0.73 and 0.93, respectively. Average number of alleles per polymorphic locus was 2.5 (range 2-4). These measurements show a high diversity, which is confirmed by the dendrogram topology. All stocks belong to the same lineage, as MN cl2 reference strain (T. cruzi II). Moreover three distinct subgroups were identified and two of them correspond to Brazilian and Bolivian zymodemes, respectively. The third subgroup, the most common in Paraguay, is related to Tulahuen stock. The large geographical distribution of some zymodemes agrees with the hypothesis of clonality for T. cruzi populations. However sample size was not adequate to detect genetic recombination in any single locality.

Key words: *Trypanosoma cruzi* - zymodemes - genetic polymorphism - clonality - Paraguay

Chagas disease is widely distributed in Latin America from the north of Mexico to the south of Argentina (Zeledón 1974). In Paraguay, like in many Latin American countries, the disease is an important public health problem, because there is a high number of *Trypanosoma cruzi* infected people and high triatomine infestation rates in dwellings (WHO 1986, Rojas de Arias 1996). Paraguay is located in the central part of South America and it is divided by the Paraguay River into two main regions: the Western or Chaco and the Eastern regions. Most of the population live in rural areas where living conditions make *T. cruzi* transmission easy. *Triatoma infestans* is the main vector and vector transmission is the most common way of acquiring the disease (Rojas de Arias 1996).

The seroprevalence rates in endemic areas of Paraguay range from 14.3% in communities in the department of San Pedro (Canese 1973), 21.6% in Paraguari, 24.9% in Cordillera (Rojas de Arias 1996) and 72.9% in an indigenous group of the Chaco region, the highest value recorded (Canese & Brice 1977).

Limited studies have analyzed the *T. cruzi* stocks collected in different areas in Paraguay. Chapman et al. (1984) related *T. cruzi* stocks isolated in the Presidente Hayes department of the Paraguayan Chaco to the Bolivian zymodeme 2. Yamasaki et al. (1990) described similar schizodeme profiles of three isolates from acute Chagas patients from distant geographical areas and related to a Brazilian strain. Mimori et al. (1992) found four distinct schizodeme groups among eleven stocks and matched two of them with the Brazilian zymodeme 2. In a previous study, Acosta et al. (1995) carried out metacyclogenesis, experimental infection in mice, lectin agglutination, parasitemic curves and morphometry with *T. cruzi* stocks from acute cases in humans, and found a great variability, especially in the biological behavior. Analysis of the geographical distribution of *T. cruzi* is often difficult because of lack of standardization of techniques, studies with reduced loci, absence of phylogenetic studies and lack of the

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same reference strains (Barnabé & Brenière 1999). The present study analyzes the isoenzyme profiles of 21 *T. cruzi* stocks mainly from the domestic cycle of different localities in Paraguay. Identical loci and similar electrophoretic conditions were used as previously to analyze large samples of *T. cruzi* from diverse origins. This allows easier identification of the taxonomic position of the Paraguayan stocks.

MATERIALS AND METHODS

Parasite stocks - Twenty one stocks from different regions of Paraguay were studied (Fig. 1). Twelve stocks belonged to human cases, eight stocks to domestic *T. infestans* and one stock to a domesticated yellow armadillo. The origin of each stock is shown in Table I. SO34 cl4 (clonets 20) and MN cl2 (clonets 39), previously typed (Brenière et al. 1998), were used as reference strains. These clones are genetically well separated and they belong to *T. cruzi* I and *T. cruzi* II groups, respectively (Anon 1999). They correspond to major clones frequently isolated in South America and they are the main clones found in the domestic Bolivian cycle (Tibayrenc & Brenière 1988, Brenière et al. 1998)

Isolation and maintenance of the stocks - The stocks were obtained by hemoculture, xenoculture and mice inoculation, maintained and amplified at 28°C in LIT medium (liver infusion tryptose) (Camargo 1964) with 50 µg/l of hemin, supplemented with 20% fetal bovine serum, 100 UI of penicillin and 100 µg of streptomycin per ml of medium.



Fig. 1: geographical location of the different Paraguayan stocks. 1, 2 and 3 correspond to the subgroups of zymodemes found.

Isoenzyme analysis - Parasite pellet was harvested in the growth peak day and washed twice with PBS at 3,000 rpm, 4°C for 10 min. The pellet was kept at -80°C until used. At the time of electrophoresis it was diluted in an equal volume of protein stabilizer solution containing 2 mM ε-amino caproic acid, EDTA and dithiothreitol.

Electrophoresis was carried out in cellulose acetate plates (Helena, Beaumont, TX) as previously described (Tibayrenc & Le Ray 1984). Thirteen enzymatic systems were studied: glucose phosphate dehydrogenase (G6PD, EC.1.1.1.49), glucose phosphate isomerase (GPI, EC.5.3.1.9), glutamate dehydrogenase NAD (GDH1, EC.1.4.1.2.), glutamate dehydrogenase NADP+ (GDH 2, EC.1.4.1.4), isocitrate dehydrogenase (IDH, EC.1.1.1.42), malate dehydrogenase (MDH, EC.1.1.1.37), malic enzyme (ME, EC.1.1.1.40.), peptidase (PEP 1, EC.3.4.11.11), peptidase (PEP 1+2, EC.3.4.11.13), phosphoglucomutase (PGM, EC.2.7.5.1), 6-phosphogluconate dehydrogenase, (6PGD, EC.1.1.1.44), diaphorase (DIA, EC.1.6.-.-), aspartate aminotransferase (GOT, EC.2.6.1.1).

The genotypes were assigned according to the profiles in the cellulose acetate plates with the already mentioned criteria of genotype numbers (Tibayrenc & Ayala 1988): band 1 corresponded to the smallest distance from the anode.

The observed heterozygosity was estimated by the following formula: $H_o = h_o / (N \times r)$, where h_o = total number of heterozygous observed, N = number of stocks and r = number of loci studied. The expected heterozygosity was estimated by the following relation: $H_e = \sum h / r$, where $h = \sum 1 - q_i^2$, and r = number of loci and q_i = frequency of the allele "i" in locus "r".

The polymorphism rate was determined by the relation: no. of polymorphic loci/no. of studied loci. The clonal diversity was measured by Whittam's (Whittam 1989) index which is equal to: $d = n (1 - \sum X_i^2) / (n-1)$, where X_i = frequency of each zymodeme and n = no. of stocks. This index measures the probability of obtaining stocks from different zymodemes when performing sampling the study population. The average of alleles per locus was calculated by the relation: $\sum a_r / r$, where a_r = no. of alleles per locus and, r = no. of studied loci.

In order to estimate the genetic divergence between zymodemes, Jaccard's phenetic distance was (Jaccard 1908): $d = 1 - (C/2N - C)$, where C = no. of common bands between two zymodemes and N = no. of total bands of both zymodemes. The UPGMA (unweighted pair-group method with arithmetic averages) (Sneath & Sokal 1973) was used to cluster the zymodemes according to

Jaccard's distances matrix. The dendrogram was built using Mac Dendro (Thioulouse 1989).

RESULTS

Genotype variability - Among the 13 enzyme systems tested, two (ME and DIA) made it possible to infer the activity of two different loci, consequently, the analysis of 15 putative loci in total was performed. Tables I and II show the multilocus genotypes of the 23 studied stocks deduced from the isoenzyme profiles obtained; stocks that showed identical isoenzymatic patterns for all the enzyme systems belong to the same zymodeme. Fifteen distinct zymodemes were identified including the two reference stocks MN cl2 and SO34 cl4 which belong to Par1 and Par15, respectively.

Among the Paraguayan stocks, four loci were monomorphic (*Mdh*, *Pep 2*, *Got*, *Dia 1*) and the polymorphism rate was 0.73. Regarding the 11 variable loci, the average number of alleles per locus was 2.5 (range 2-4). Several patterns observed for *Gpi*, *Idh*, *Pgm* and *6pgd* loci were characteristic of heterozygotes, and the observed heterozygosity was 0.17. The average value of expected heterozygosity was 0.34; a deficiency of heterozygotes was hence apparent. The number of stocks belonging to a single zymodeme range from

1 to 6 for Par 5 and the probability of sampling two different zymodemes in the population measured by the Whittam's index was 0.93.

Phenetic relationships - The dendrogram (Fig. 2) based on the Jaccard's distance matrix showed two upper branches which included a reference stock each. They correspond to the two principal groups of *T. cruzi* previously described (Tibayrenc 1995, Brenière et al. 1998). All the Paraguayan stocks belong to the *T. cruzi* II as MN cl2 reference stock. Moreover, *T. cruzi* II, presented three separated branches (subgroups 1 to 3) with distances ranging from 0.35 to 0.65 between them (Fig. 2). Four stocks were closely related to Mn cl2 reference stocks (subgroup 1) which belong to the most common zymodeme (clonets 39) found in the domestic Bolivian cycle. Except the Arma 134 (the only stock isolated from a sylvatic mammal) all the stocks of subgroups 1 and 2 presented variable heterozygote patterns for the *Gpi* locus and those of subgroups 3 had the same homozygous profile (genotype 2/2, *Gpi* locus). The distribution of zymodemes according to their hosts and geographical origin is shown in Figs 1 and 2. An absence of specific geographical distribution of the zymodeme groups was observed, since for example zymodemes belonging to subgroup 2 were found

TABLE I
Origin of the *Trypanosoma cruzi* stocks examined

Stocks	Department	Host	Zym	Sub-group
HV	Caaguazú	Human (acute)	Par 9	2
ROFI	Central	Human (acute transfusional)	Par 13	3
JM	Central	Human (acute transfusional)	Par 14	3
RN Lezcano	Central (mother from San Pedro)	Human (congenital)	Par 1	1
JAG	Cordillera	Human (acute)	Par 13	3
DG	Paraguarí	Human (indeterminate)	Par 10	3
Xc 116	Paraguarí	<i>Triatoma infestans</i>	Par 2	1
FALE	Paraguarí	Human (indeterminate)	Par 3	1
GS	Presidente Hayes	Human (acute)	Par 11	3
Xc ILSE	Presidente Hayes	Human (acute)	Par 5	2
LAURA	Presidente Hayes	Human (acute)	Par 5	2
Coraf 6	Presidente Hayes	<i>Triatoma infestans</i>	Par 5	2
Coraf 14	Presidente Hayes	<i>Triatoma infestans</i>	Par 5	2
Lengua 15	Presidente Hayes	<i>Triatoma infestans</i>	Par 5	2
Coraf 7	Presidente Hayes	<i>Triatoma infestans</i>	Par 6	2
Coraf s/n	Presidente Hayes	<i>Triatoma infestans</i>	Par 7	2
Lengua 16	Presidente Hayes	<i>Triatoma infestans</i>	Par 7	2
Coraf 18	Presidente Hayes	<i>Triatoma infestans</i>	Par 8	2
EV	San Pedro	Human (chronic)	Par12	3
ARMA 134	San Pedro	<i>Euphractus sexcintus</i> (yellow armadillo)	Par 4	1
EMI	San Pedro	Human (acute)	Par 5	2
SO34 cl4 ^a	Potosi (Bolivia)	<i>Triatoma infestans</i>	Par 15	
MN cl2 ^a	IV Region (Chile)	Human (chronic)	Par 1	1

a: laboratory clones 20 (SO34 cl4) and 39 (MN cl2) used as references (Tibayrenc & Ayala 1988).

TABLE II
Multilocus genotypes of the 15 *Trypanosoma cruzi* zymodemes identified with 15 loci

Zym	NB	G6pd	Gpi	Gd nad	Gd nadp	Idh	Mdh	Me1	Me2	Pep1	Pep2	Pgm	6Pgd	Got	Dia1	Dia2
Par 1	2	2/2	1/4	1/1	1/1	2/2	1/1	2/2	2/2	3/3	2/2	2/4	1/3	1/1	1/1	1/1
Par 2	1	2/2	1/4	1/1	1/1	2/3	1/1	2/2	2/2	3/3	2/2	2/4	1/3	1/1	1/1	1/1
Par 3	1	2/2	1/4	1/1	1/1	2/3	1/1	2/2	3/3	3/3	2/2	2/4	99 ^a	1/1	1/1	1/1
Par 4	1	3/3	4/4	1/1	1/1	2/2	1/1	2/2	2/2	3/3	2/2	2/4	1/3	1/1	1/1	1/1
Par 5	6	2/2	3/4	1/1	2/2	2/2	1/1	2/2	2/2	2/2	2/2	2/4	1/1	1/1	1/1	2/2
Par 6	1	2/2	3/4	1/1	2/2	2/3	1/1	2/2	2/2	2/2	2/2	2/4	1/3	1/1	1/1	2/2
Par 7	2	1/1	3/4	1/1	2/2	2/2	1/1	2/2	2/2	2/2	2/2	2/4	1/3	1/1	1/1	2/2
Par 8	1	2/2	3/4	1/1	2/2	2/2	1/1	3/3	3/3	2/2	2/2	2/4	1/3	1/1	1/1	2/2
Par 9	1	2/2	1/4	1/1	2/2	2/2	1/1	2/2	2/2	2/2	2/2	2/4	1/1	1/1	1/1	2/2
Par 10	1	1/1	2/2	2/2	3/3	2/3	1/1	2/2	2/2	2/2	2/2	4/4	1/1	1/1	1/1	3/3
Par 11	1	1/1	2/2	2/2	3/3	2/3	1/1	3/3	2/2	2/2	2/2	4/4	1/1	1/2	1/1	3/3
Par 12	1	1/1	2/2	2/2	3/3	2/3	1/1	3/3	2/2	2/2	2/2	4/5	1/1	1/1	1/1	3/3
Par 13	2	1/1	2/2	2/2	3/3	2/3	1/1	3/3	2/2	3/3	2/2	4/5	1/1	1/1	1/1	3/3
Par 14	1	1/1	2/2	2/2	2/2	2/3	1/1	3/3	2/2	3/3	2/2	4/4	1/3	1/1	1/1	1/1
SO34	1	3/3	5/5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	1/1	1/3	2/3	2/2	1/1	1/1

Zym: zymodeme; NB: number of stocks; a: not done

in Presidente Hayes, San Pedro and Caaguazú departments, and zymodemes of the three subgroups were observed in the San Pedro department. Regarding the host, all the studied stocks (except one) were from human cases and *T. infestans* which is the principal vector in Paraguay. Therefore all these stocks can be considered as part of the domestic cycle. As expected similar zymodemes and closely related zymodemes could be found in *T. infestans* and human (eg. Par 5) but zymodemes of subgroups 3 were only isolated from humans. Nevertheless, such a host specificity must be verified with larger samples of stocks.

DISCUSSION

The isoenzyme technique has been widely used to characterize *T. cruzi* populations of different countries belonging to domestic and sylvatic cycles (Miles et al. 1980, Tibayrenc & Miles 1983, Carneiro et al. 1991, De Luca D'oro et al. 1993, Lewicka et al. 1995, Brenière et al. 1997) but limited data are available from Paraguayan stocks. The comparison of isoenzyme data with other genetic markers gave congruent results and showed the division of *T. cruzi* taxon into two principal groups (Tibayrenc 1995, Souto et al. 1996, Brisse et al. 1998, Brenière et al. 1998). According to the analysis of MLEE data (22 loci) and RAPD (10-mer primers) together *T. cruzi* I and *T. cruzi* II present high bootstrap values (97% and 93% respectively), a strong support of the *T. cruzi* division in two lineages; the SO34 cl4 stock belongs to *T. cruzi* I and MN cl2 to *T. cruzi* II. In the present study, the isoenzyme analysis of 15 loci allowed the classification of the 21 Paraguayan stocks into 14 zymodemes and the estimation of the degree of genetic differentiation among them. All the stocks were clustered in an upper branch with the MN cl2 stock used as control and none of them were genetically related to the SO34 cl4 stock. Moreover, the comparison of the distances with previous data show that the Paraguayan stocks can be considered as belonging to *T. cruzi* II. Nevertheless, among the stocks, genetic diversity is still important, and three subgroups were observed. Previous analysis also showed that group *T. cruzi* II presents a high genetic diversity and Tibayrenc (1998) proposed five subgroups. The relationship between these subgroups and the three Paraguayan subgroups is more difficult to interpret. Nevertheless, it is worth noting that the stocks belonging to subgroups 1 and 2 have heterozygous profiles for *Gpi*, *Pgm* and *6Pgd* loci, hence they should correspond to two different zymodemes named Z39 and Z43 by Tibayrenc (1998): Z39 include MN cl2 stock and Z43 include the laboratory reference strain Tulahuén. The remaining Paraguayan stocks are clustered apart and

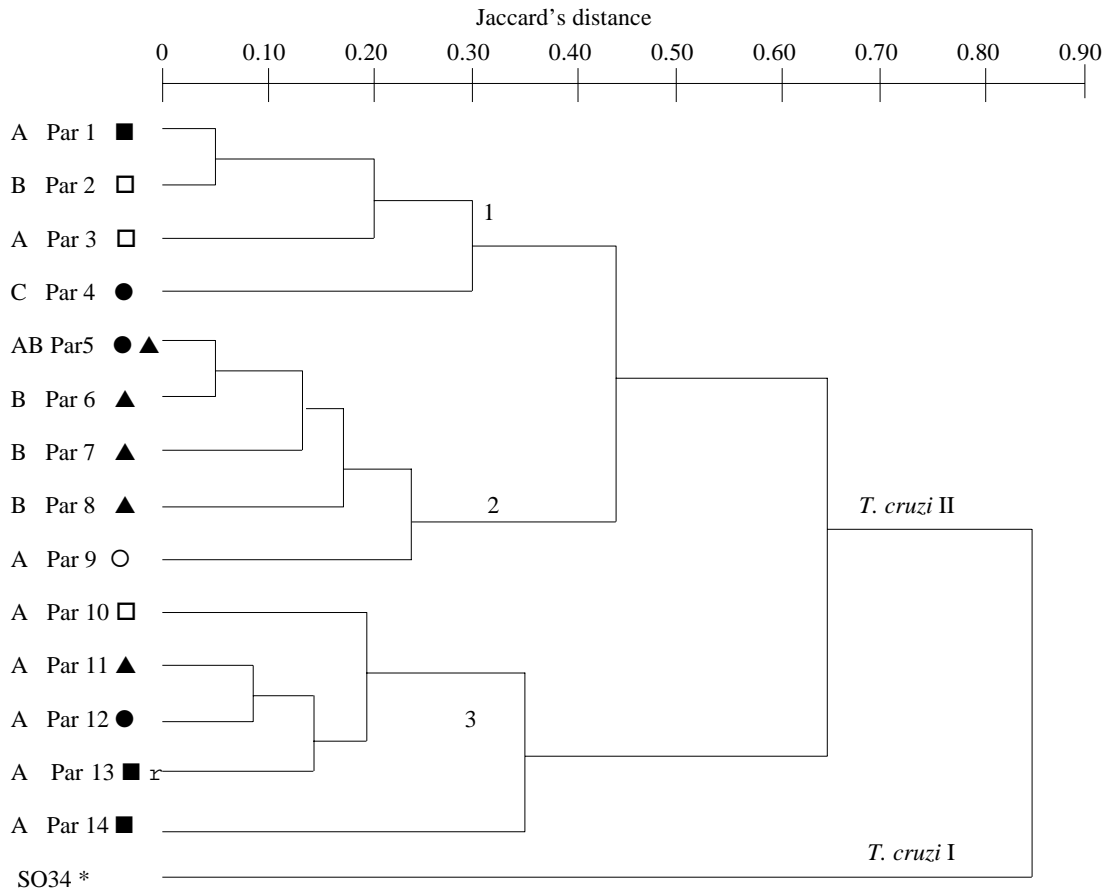


Fig. 2: dendrogram constructed by the hierarchical ascending method UPGMA from Jaccard's distance matrix (15 loci), for 21 *Trypanosoma cruzi* zymodemes. Host and geographical origins are indicated for each zymodeme. A: human, B: *Triatoma infestans*; C: *Euphractus sexcintus* (armadillo); ▲: Presidente Hayes; ●: San Pedro; r: Cordillera; ■: Central; O: Caaguazú; □: Paraguarí. 1, 2 and 3 correspond to the subgroups ;*: reference stock (SO34)

did not present heterozygous patterns (subgroup 3) but gave the same profile as Esmeraldo cl3 stock (Brazilian Z2 reference) at *Gpi* and *6Pgd* loci which was previously characterized by Brenière et al. (1998). Previous analysis of Paraguayan *T. cruzi* stocks by isoenzyme (Chapman et al. 1984) and restriction enzyme analysis of kinetoplast DNA (Yamashi et al. 1991, Mimori et al. 1992) showed that the stocks shared most features of the Bolivian Z2 and Brazilian Z2 *T. cruzi* stocks. More recent works showed that these zymodemes belong to *T. cruzi* II (Brenière et al. 1998), but are clustered apart in distinct subgroups (Tibayrenc & Miles 1983, Barnabé & Brenière 1999): Bolivian Z2 and Brazilian Z2 belong to Z39 and Z32, respectively, according to Tibayrenc (1998). The present and previous results agree with the presence in Paraguay of stocks genetically related to: (i) the Brazilian Z2 (subgroup 3) (ii) Bolivian stocks

pertaining to one of the most frequent clone circulating in the Bolivian domestic cycle (clonets 39) (Brenière et al. 1995, 1998) (subgroup 1); and (iii) Tuluhen reference stock (subgroup 2). The stocks belonging to the latter were the most abundant in Paraguay (50% of the studied sample).

High genetic variability in the studied population was confirmed by the index of polymorphism and Whittam's which are similar to others found in other countries (Tibayrenc & Ayala 1988, De Luca D'Oro et al. 1993, Brenière et al. 1997).

The criteria of clonality for *T. cruzi* populations has been widely described in several studies (Tibayrenc & Desjeux 1983, Tibayrenc et al. 1984, Tibayrenc & Ayala 1988). The results obtained in this study agree with this hypothesis. A stock isolated from a human case of San Pedro department presented the same genotype as stocks from human and triatomines of Presidente Hayes depart-

ment. These two regions are geographically separated by the Paraguay River (see Fig. 1). In the same way, the stock RN Lezcano showed the same profiles as MN cl2 reference strain isolated in other countries and at different times. Also, the possibility of contamination was minimal because the parasites were processed separately by different laboratories. The fact of finding stocks with the same profile in geographically distant areas and isolated in different moments confirms the ubiquitous feature of these zymodemes which is one of the clonality criteria (Tibayrenc & Ayala 1988). The difference between expected and observed heterozygosity values also supports the clonality. Some studies suggest genetic exchanges in this organism (Bogliolo et al. 1996, Carrasco et al. 1996). The clonal model does not exclude the possibility of the occurrence of genetic exchange. It is only that the exchange is not frequent enough for the current genotypes or zymodemes to be considered unstable or transient entities.

The identification of the clonal types of circulating *T. cruzi* and their geographical distribution are important for the study of Chagas disease. Based on clonal types main and secondary species of vector can be identified, the relationship between wild and domestic cycles can be evaluated, reservoirs can be identified and the observed pathologies can be compared (Barnabé & Brenière 1999).

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