Molecular karyotype and schizodeme analyses of *Trypanosoma cruzi* stocks from Chilean triatomines

J. VENEGAS, S. ORTIZ, S. MUNOZ *and* A. SOLARI*

Department of Biochemistry, Faculty of Medicine, University of Chile, Casilla 70086, Santiago 7, Chile

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SUMMARY

Forty-one *Trypanosoma cruzi* stocks isolated from the Chilean vectors *Triatoma infestans* and *Triatoma spinolai* were characterized by pulse-field gel electrophoresis and Southern blotting with the cruzipain gene, and by schizodeme analysis of kinetoplast DNA with *Eco*RI and *Msp* I. Seven parasite groups were found by molecular karyotype which correlate with schizodeme and multilocus enzyme electrophoresis, supporting the concept of clonal propagation for *Trypanosoma cruzi*. A predominant *T. cruzi* stock was isolated from domiciliary *T. infestans* in several geographical areas of Chile. In contrast, other frequently found genotypes were circulating in the sylvatic and domestic transmission cycles of specific geographical areas. The greatest heterogeneity of *T. cruzi* stocks was found among sylvatic *T. spinolai* where at least 4 genotypes were obtained from a sample of 16 *T. cruzi* stocks.

Key words: karyotype, schizodemes, *Trypanosoma cruzi*, *T. infestans*, *T. spinolai*.

INTRODUCTION

The haemoflagellate parasite *Trypanosoma cruzi* is the agent of Chagas' disease in South and Central America (Walsh, 1984). This is a pleomorphic entity in which different manifestations can occur during the acute and chronic phases of the disease. Epidemiological evidence suggests that parasite factors could explain clinical variability of Chagas' disease in different geographical endemic areas (Brener, 1973). *T. cruzi* has a broad host range infecting wild and domestic mammals, which provide large reservoirs of parasites. The parasites are transmitted by blood-sucking reduviid bugs of different species depending on the geographical area. The genetic variability of *T. cruzi* populations in Chile has been explored by isoenzyme characterization, as well as by kinetoplast DNA restriction fragment length polymorphism (kDNA RFLP), or schizodeme analysis, and DNA hybridization studies (Miles *et al*. 1984; Apt *et al*. 1987; Carreno *et al*. 1987; Macina *et al*. 1987; Solari *et al*. 1991, 1992). Natural population genetic studies with isoenzymatic markers of an extensive *T. cruzi* sample revealed a clonal structure: the zymodemes described by 15 isoenzyme loci can be equated with natural clones or families of genetically closely related populations (Tibayrenc *et al*. 1986; Tibayrenc & Ayala, 1988).

The kDNA is a distinctive feature of the order Kinetoplastida which includes trypanosomes, leishmanias, leptomonas, phytomonas, herpetomonas and crithidias. This kDNA accounts for 20% of the DNA of the cell and is made up by 2 components: the maxi-circle, which encodes mitochondrial genes, and the mini-circle, which encodes small RNA guide transcripts, and edits the mitochondrial transcripts (Schlomai, 1994).

Size fractionation of chromosomes by pulsed-field gel electrophoresis (PFGE) has been a useful tool for studying the genotype of natural *T. cruzi* populations (Henriksson, Pettersson & Solari, 1993). The results from these PFGE experiments indicate a high variability of chromosome size between different *T. cruzi* stocks. In this study, in order to analyse *T. cruzi* populations from insect vectors found in different geographical areas of Chile, schizodeme analysis was used in parallel with molecular karyotype. The aim of this work was to distinguish *T. cruzi* populations from different insect vectors and to test the clonal reproduction hypothesis for *T. cruzi* using mitochondrial and nuclear DNA markers. The study also establishes the heterogeneity of genotypes and their frequency in both the sylvatic and domestic transmission cycles of Chile.

MATERIALS AND METHODS

Parasites

T. cruzi epimastigotes from recently isolated stocks and cryopreserved isolates were grown in a liquid medium (Diamond, 1968). Table 1 lists the information on parasite host origin, geographical location and isoenzyme analyses. Two *T. cruzi* stocks (LQ and LGN) with unique genotypes isolated from humans were included in this study. Both stocks were screened in a field study of 98 cases (Apt *et al*. 1987). The *T. rangeli* stock (LDG) was from a Colombian infected subject (Henriksson *et al*. 1996).

* Corresponding author. Tel: +678 60 62. Fax: +735 55 80.

Table 1. Details of *Trypanosoma* spp. host origin, geographical location and isoenzyme analyses

Stock	Locality	Geographical region	Host	Parasitic groups
vTV-E	Iquique	I Región	*T. infestans*	II
vTV-T	Iquique	I Región	*T. infestans*	Ia
vMV3	Iquique	I Región	*T. infestans*	Ia
vGM	Iquique	I Región	*T. infestans*	Ib
vFRA2	Francia	I Región	*T. infestans*	IV
vQUII	Quistagama	I Región	*T. infestans*	Ia
v195	San Pedro	II Región	*T. infestans*	IV
v111, v115, v121	San Pedro	II Región	*T. infestans*	Ia
LQ	La Isla	IV Región	Human	Ia
LGN	Illapel	IV Región	Human	Ic
v2X	Monte Grande	IV Región	*T. infestans*	II
v1738	Las Ramadas	IV Región	*T. infestans*	III
v85, v98, v1660 v1672, v1759	Campanario	IV Región	*T. infestans*	II
vOV1	Ovalle	IV Región	*T. infestans*	II
vOV6	Ovalle	IV Región	*T. infestans*	II
sp153	Ramadilla	IV Región	*T. spinolai*	III
v1649, spAI, spAII	Flor del Valle	IV Región	*T. spinolai*	Id
spCOMB2, spCOMB1	Combarbalá	IV Región	*T. spinolai*	Ia
v213	Flor del Valle	IV Región	*T. spinolai*	Ia
spI, CHI22, WALLF	Flor del Valle	IV Región	*T. spinolai*	Ib
sp54, sp73	Bellavista	IV Región	*T. spinolai*	Ic
sp104, sp130, sp137, sp142, sp161	Ramadilla	IV Región	*T. spinolai*	Ia
v89	Campanario	IV Región	*T. spinolai*	I
v101-T	Campanario	IV Región	*T. spinolai*	Ib
vTM	Santiago	Metropolitan Reg.	*T. infestans*	II

Agarose block preparations

T. cruzi epimastigotes from the different stocks were prepared as described (Henriksson *et al*. 1990). From 1 to 20×10^6 epimastigotes were used in each well.

Schizodeme analysis

kDNA parasites were obtained according to conditions previously described (Goncalves, Nehme & Morel, 1984). kDNA samples were digested to completion with an excess of restriction endonuclease according to the manufacturer's buffer conditions. The digestion products were electrophoresed in a 4·5–10 % polyacrylamide gel gradient and further stained with silver nitrate, as previously described (Goncalves, Nehme & Morel, 1990).

Pulsed-field gel electrophoresis

All PFGE separations were carried out in 1 % agarose gels. The running buffer (0·5 × TBE) was maintained at 14 °C. All separations were performed using the BioRad Chef II. The standard parameter used was a time-ramping from 57·8 sec to 169·3 sec pulse time at 6 V/cm during 34 h 11 min, which gave an optimal separation of DNA molecules between 350 and 1800 kb. Chromosomes from *S. cerevisiae* were used as molecular weights markers. DNA was transferred to nylon membranes using standard methods (Southern, 1975) after soaking gels in 0·25 M HCl for 15 min to nick the DNA and effect further neutralization (Henriksson *et al*. 1990).

PFGE and hybridization studies

The terminal extension of the cruzipain gene was chosen as the probe based on the criteria of known variable hybridization patterns in previous studies (Henriksson *et al*. 1990). The probe was digoxigenin labelled using the random priming method (Boehringer) and, after hybridization, the filters were washed at high stringency conditions (0·1 × SSC, 0·1 % SDS at 65 °C).

RESULTS

Schizodeme analysis of T. cruzi *isolates*

The kDNA RFLP pattern obtained after digestion with both restriction endonucleases (*Msp* I and *Eco*R I) permitted a firm and clear classification of the studied isolates into 4 major groups. Information

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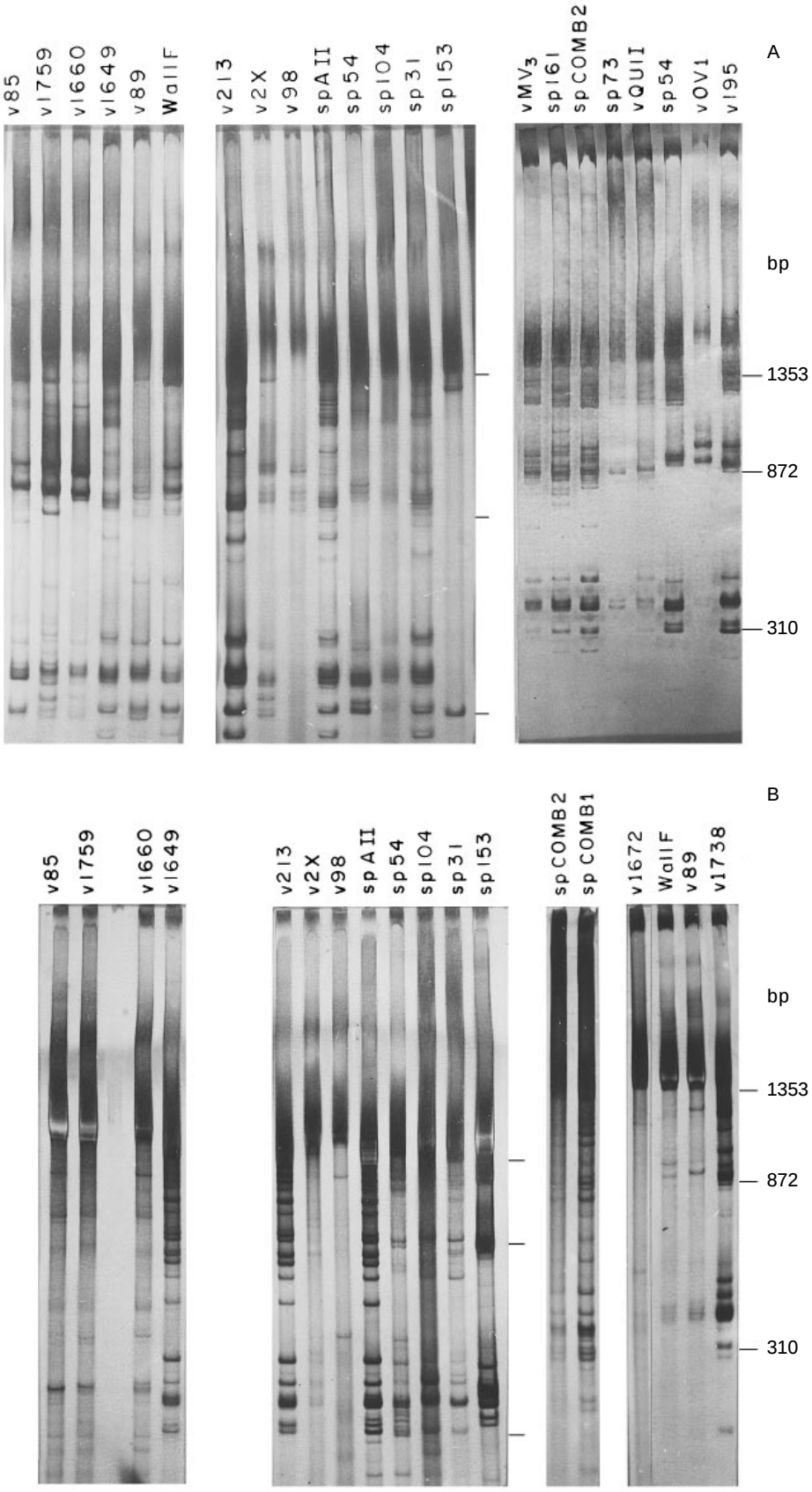

Fig. 1. (A) Restriction patterns of *Trypanosoma cruzi* using *Eco*R I. The identities of the samples are indicated on top of the gel in all panels. Numbers to the right refer to size of molecular weight markers. (B) Restriction patterns of *T. cruzi* isolates using *Msp* I. The identity of samples and estimated DNA sizes are as described in (A).

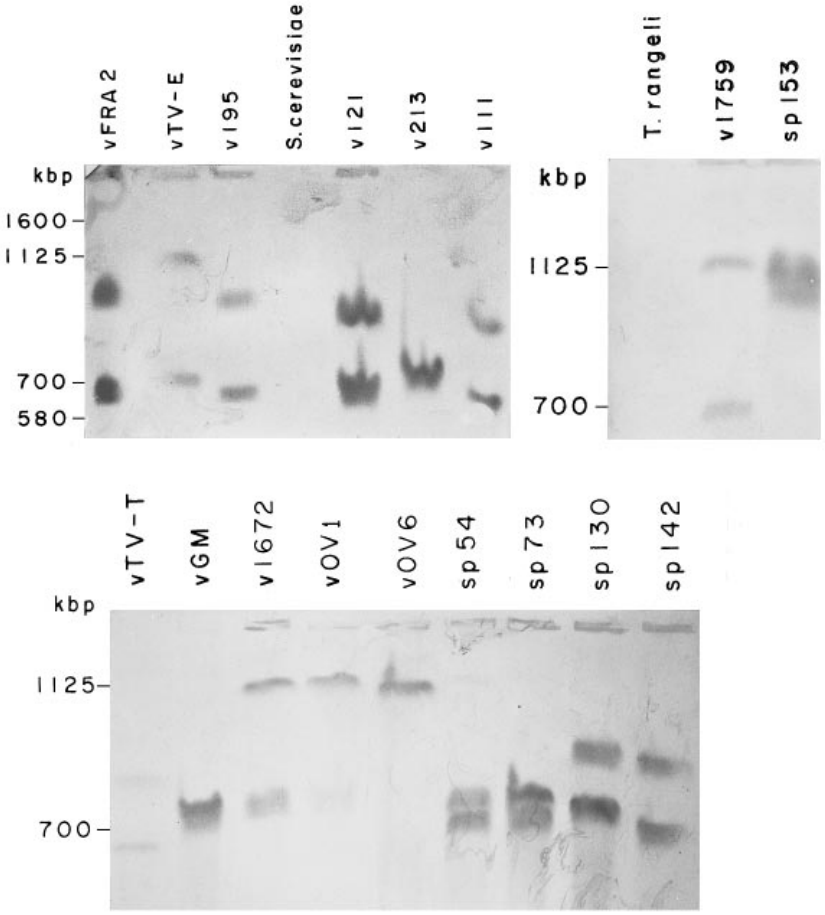

Fig. 2. Some examples of PFGE gels and hybridization patterns revealed by the cruzipain probe. The hybridization signal in the upper part of the figure corresponds to material trapped in the agarose plugs retained in the slots. Numbers to the left refer to sizes in kbp estimated from *S. cerevisiae* chromosomes.

about *T. cruzi* samples and parasite groups is summarized in Table 1. This grouping arises from a qualitative genotypic interpretation based on the similarities between the restriction patterns of kDNA samples digested with *Eco*R I or with *Msp* I. Group I is composed of isolates mainly from *T. spinolai* (sylvatic transmission cycle of Regions III and IV) and *T. infestans* (domestic and sylvatic transmission cycle) from the north of Chile (Regions I and II). Selected kDNA samples were digested with *Eco*R I (Fig. 1A) or with *Msp* I (Fig. 1B). The parasites from Group I give very heterogeneous banding patterns with both restriction endonucleases. Some representative isolates of these groups are: vGM, v213, vMV3, spAII, sp54 and sp161. Group II is composed of isolates from *T. infestans* of the domestic transmission cycle all over the country, giving *Eco*R I banding patterns concentrated on the 872 bp size and few bands using *Msp* I. Some isolates of this group are v2X, v85, v1660, and v98. Group III was detected only in 2 isolates (sp153 and v1738), which are characterized by the abundant bands generated by *Msp* I and poor banding patterns with *Eco* RI. Finally, the group IV described here is composed of parasites found exclusively in *T. infestans* of the I and II regions (v195 and vFRA2). This group gives an *Eco*R I profile similar to parasite group I and its *Msp* I profile is almost identical to that of group II.

Hybridization to PFGE-fractionated T. cruzi *chromosomes*

PFGE-separated chromosomes were hybridized with the cruzipain probe as described in the Materials and Methods section (Fig. 2). There were some variations in the intensities between the hybridization signals and retarded chromosomal migrations in different lanes due to either variations in the amount of DNA loaded in each lane or to different number of copies of the sequences in the different stocks. The pattern obtained with the cruzipain probe is very informative, because it gives 1 or 2 hybridizing chromosomal signals of different molecular sizes. At least 7 different patterns can be described. The 28 isolates belonging to the major schizodeme group I are subclassified into 4 subgroups (Ia, Ib, Ic and Id) based on the molecular karyotype banding (see Table 1). The fifth karyotype group is composed of 10 identical isolates corresponding to the schizodeme group II. The sixth karyotye group is characterized by the unique

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banding pattern which correlates to the schizodeme group III. The seventh karyotype group which corresponds to the schizodeme group IV emerges from this study with banding profiles very similar to the schizodeme group II. Finally a new profile was obtained for the vOV1 and vOV6 isolate which is more similar to group II than to any other group.

No hybridization was detected with the *T. rangeli* isolate and *S. cerevisiae*.

DISCUSSION

Population genetic studies with a huge number of *T. cruzi* samples from different endemic areas of America, using isoenzyme analysis, led others to suggest that *T. cruzi* has a clonal rather than a sexual reproduction (Tibayrenc & Ayala, 1988; Tibayrenc, Kjellberg & Ayala, 1990). However, population genetic studies with sylvatic *T. cruzi* stocks suggest that genetic exchange does contribute to the generation of diversity in *T. cruzi* (Carrasco *et al*. 1996).

Here, 2 methodological approaches were performed to study *T. cruzi* populations. The first method was the kDNA RFLP or schizodeme analysis, which is based on the heterogeneous sequence of the mini-circles. The second was based on the molecular karyotype using the 3′ terminal extension of the cruzipain gene as probe. Previous studies allowed clustering of the Chilean *T. cruzi* stocks into 3 major groups of similar schizodemes corresponding to the zymodemes Z1, Z2bol and Z2bra, but with great variability within each group (Carreno *et al*. 1987; Solari *et al*. 1992).

Several genotypes have been found with this sample. Group II is very homogeneous and corresponds to the classical zymodeme (Z2bol), or to the parasite clone 39 (Tibayrenc & Ayala, 1988). However, when these isolates are studied at 19 genetic loci they are subdivided into the genotypes 26–28, 32 and 33 (Neubauer, 1992). The isolates studied here (v1672, v1759, and vTV-E) pertain to the genotype 27 and showed an identical molecular karyotype. Parasites of group IV belong to the genotype 29 and display a very similar molecular karyotype pattern to the previous group, with 2 shorter chromosomes carrying the cruzipain genes. The third group of parasites belongs to the classical zymodeme Z2bra (Miles *et al*. 1984), clones 32 and 33 (Tibayrenc & Ayala, 1988). However, when studied at 19 genetic loci they split into genotypes 20–23 (Neubauer, 1992). Finally, the first group of *T. cruzi* populations is the most heterogeneous one. This parasite group is in agreement with the zymodeme Z1 previously described (Miles *et al*. 1984). Meanwhile the subgroup Ia corresponds to the genotypes 8, 10–13 and 24, the other parasites grouped here as Ib, Ic and Id correspond to the genotypes 6, 18 and 2 respectively (Neubauer, 1992). It is worth pointing out that all the *T. cruzi* stocks classified as group I are genetically related, especially the subgroups Ib and Id, and very different from the genotypes belonging to the groups II, III and IV (Neubauer, 1992).

The results obtained here are similar to others of the *T. cruzi* classification obtained with several genetic markers. Studies with isoenzymatically characterized *T. cruzi* stocks and clones revealed a strong correlation between schizodemes, isoenzymes, Southern blotting with kDNA probes, molecular karyotype and RAPD (Carreno *et al*. 1987; Macina *et al*. 1987; Tibayrenc & Ayala, 1987; Henriksson *et al*. 1983; Tibayrenc *et al*. 1993). The association between highly polymorphic patterns among pairs of stocks as described here provides classical evidence of clonality since it implies that specific DNA markers in the kinetoplast are jointly transmitted with specific nuclear markers. Such an absence of recombination in nuclear DNA would not be expected in sexually reproduced organisms. The demonstration of a strong correlation between classification of natural populations of *T. cruzi* by independent molecular markers supports this conclusion.

The present study and others demonstrate that a ubiquitous parasite type is found in domestic environments in a huge geographical extension of Chile and Argentina (Miles *et al*. 1984; Apt *et al*. 1987; Macina *et al*. 1987; Solari *et al*. 1992; De Luca D'Oro *et al*. 1993; Munoz *et al*. 1994; Gonzalez *et al*. 1995). This genotype corresponds mainly to clone 39 or genotype 27, and may be of epidemiological and medical significance given its high frequency on the South Cone of America. For this reason it is worthwhile studying several aspects of this particular *T. cruzi* type, such as pathological relevance, target for vaccine and drug development.

On the other hand very different and varied *T. cruzi* genotypes are found in the sylvatic transmission cycles of the IV Region (*T. spinolai*) and domestic habitats of the I and II Regions in the extreme north of Chile, where *T. infestans* is the exclusive vector. Parasite groups Ia and Ib circulate in the above described habitats and groups Ic and Id are also distributed in the sylvatic transmission cycle of the IV Region. In conclusion, the particular distribution of parasite groups Ia, Ib, Ic and Id could be explained by the exclusive presence of the sylvatic vector *T. spinolai* in the III and IV Regions, which are geographically separated from the I and II Regions by the Atacama Desert. Parasite group II, clone 39 or genotype 27 on the other hand is better adapted to *T. infestans* in the domestic environments of the most endemic areas (III and IV Regions), and explains the epidemiology of Chagas' disease in Chile which is mainly caused by the infection of humans with this genotype.

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