Trypanosoma cruzi isolates from Chile are heterogeneous and composed of mixed populations when characterized by schizodeme and Southern analyses

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SUMMARY

In total, 61 Chilean isolates of *Trypanosoma cruzi*, were analysed using schizodeme and Southern analysis, using as probes the highly variable regions of minicircles from cloned parasites. Isolates were collected and amplified from domestic and wild triatomines, and from infected subjects in all the endemic areas of Chile. Three major parasite genotypes could be detected in the domestic transmission cycle, whilst 1 major *T. cruzi* genotype is circulating in the wild transmission cycle. Schizodeme analysis suggested that *T. cruzi* isolates are mixed populations, whereas the Southern analyses detected only 3 mixed isolates using 4 selected minicircle segments as probes.

Key words: Southern analysis, schizodemes, mixed populations, *Trypanosoma cruzi*.

INTRODUCTION

Strains, isolates and clones of *Trypanosoma cruzi*, the infective agent of Chagas disease, differ in several biological and biochemical properties (Revollo *et al*. 1998). These differences include their infectivity in host cells, virulence and pathogenesis in animal models, drug sensitivity, tissue tropism, morphology, and antigenic composition. This has important implications for future chemotherapeutic or immunological approaches to treatment of the disease, since not all *T. cruzi* parasites may be susceptible to a given drug (Revollo *et al*. 1998).

Although 3 main isoenzymatic groups or zymodemes (Z1, Z2 and Z3) were originally described (Miles *et al*. 1980), it is now clear that great heterogeneity is present within each group, suggesting a complex multiclonal origin of *T. cruzi* populations distributed in 2 main phylogenetic lineages named by international consensus as *T. cruzi* I and *T. cruzi* II (Anonymous, 1999). *T. cruzi* I corresponds to Z1 (Barnabe, Brisse & Tibayrenc, 2000), and it has been proposed that *T. cruzi* II consists of 5 sublineages, one corresponding to Z2, and one to Z3 (Brisse, Barnabe & Tibayrenc, 2000).

Zymodeme characterization often involves isolation of the parasite from the host by xenodiagnosis (or culture) and amplification by serial passage in culture. These procedures are necessary when large numbers of parasites are needed for isoenzyme analysis. For this reason originally mixed parasite populations can generate divergent parasite characterization results if particular *T. cruzi* clones are selected during their bulk growth.

Given the association between zymodemes and schizodemes (groups of parasites defined by RFLP patterns of kDNA), characterization of parasites can be achieved by analysis of kDNA by hybridization tests (Macina *et al*. 1987; Tibayrenc & Ayala, 1987). Minicircles, one of the two components of the mitochondrial DNA of trypanosomatids (Englund, Hajduk & Marini, 1982), are known to evolve rapidly (Stuart, 1983), mainly due to accumulation of point mutations (Macina *et al*. 1986). Due to this rapid sequence divergence, schizodemes from most isolates and strains differ to some extent. *T. cruzi* minicircles have two kinds of sequence elements; a conserved region of 118 bp repeated 4 times per molecule and 4 variable and divergent regions perfectly intercalated among the repeats (Macina *et al*. 1986; Degrave *et al*. 1988). Conserved regions seem to be similar in sequence, among the approximately 30 000 minicircles present per parasite as well as among minicircles of different isolates (Morel *et al*. 1980; Macina *et al*. 1986). On the other hand, divergent regions are different not only among molecules that belong to different minicircle sequence classes, but also within minicircles of a single parasite clone (Frasch, Sanchez & Stoppani, 1984; Macina *et al*. 1985). A low level of intraminicircle sequence similarity was also observed in the variable region by Degrave *et al*. (1988), but this similarity did not extend to between isolates.

Amplification of highly variable regions of minicircles (HVRm) of kDNA by PCR and hybridization

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with labelled probes, also permits the identification of the zymodeme (Veas *et al*. 1991).

In this paper we analysed several *T. cruzi* parasites obtained in Chile from human hosts and insect vectors, with the aim of estimating how much parasite diversity is present and whether kDNA restriction fragment length polymorphism and DNA probes using HVRm could be used to group natural isolates. Another aim was to estimate how many of the *T. cruzi* isolates from nature are mixed populations using two methods of different discriminatory power, to mention schizodeme and Southern analysis.

MATERIALS AND METHODS

Identification of Trypanosoma cruzi *isolates*

Isolates were analysed from all the geographical regions in Chile where Chagas disease occurs (Table 1). Isolates from the northern political regions of Chile, I and II (18°S–23°S); were from *T. infestans* and chronic chagasic patients. Isolates from regions III and IV (25°S–31°S), where the disease is most endemic in Chile, also included stocks from the vector *Mepraia spinolai*. Isolates from the Southern region V and the Metropolitan region (31°S–34°S) were from chronic patients with *T. infestans* and *M. spinolai*. The experimental results obtained after hybridization of the digested kDNA from each isolate with DNA probes are shown in Figs 1 and 2, and summarized in Table 1.

Parasites were obtained from chagasic patients by xenodiagnosis with *Triatoma infestans* followed by cultivation of the parasites in liquid medium 1 (Diamond, 1968), from the whole digestive tract or faeces of the insect vector.

Schizodeme analysis

Kinetoplast DNA of parasites were obtained as previously described (Sanchez *et al*. 1993). 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 stained with silver nitrate, as previously described (Goncalves, Nehme & Morel, 1990).

Mouse infection

Metacyclic trypomastigote forms were obtained by metacyclogenesis from epimastigotes grown in Diamond's medium supplemented with calf serum as described (Wallace *et al*. 2001). These trypomastigote forms were inoculated into irradiated (450 Rad.) Balb/c mice weighing 20–22 g. From these mice normal male mice were infected with each isolate by intraperitoneal inoculation of blood trypomastigote forms collected at the peak of parasitaemia. Several passages (4–6) were used to adapt each isolate to mice, producing a patent infection. The parasites were re-isolated by haemocultivation in Diamond's medium supplemented with 10% foetal calf serum, and then grown to a final yield of approximately 10^9 cells in exponential growth for schizodeme re-analysis.

kDNA purification, DNA probes and hybridization conditions

HVRm from 4 different *T. cruzi* clones were used as probes to determine by Southern analysis with total kDNA the parasite populations infecting insect vectors and chagasic patients in Chile. Construction of subspecific HVRm probes for *T. cruzi* clones was performed as previously described (Veas *et al*. 1991). These fragments, close to 250 bp in size, do not contain the constant region that can cross-hybridize with minicircles of other *T. cruzi* genotypes (Solari *et al*. 2001). Each probe presents a great variety of different molecules, even though they are very similar in sequence since minicircle heterogeneity exists within a single parasite clone (Macina *et al*. 1986). This is an advantage over using cloned fragments as probes because in the latter cases only molecules of the same minicircle sequence class are detected (Macina *et al*. 1985). Thus it is possible, using HVRm probes, to detect similarities among genetically related groups of parasites as previously demonstrated (Breniere *et al*. 1998). A very good correspondence between hybridization patterns and lineages determined by isoenzyme characterization has been found (Veas *et al*. 1991; Solari *et al*. 2001). Therefore the method is validated for epidemiological purposes, using total kDNA as probes. This association also has been found previously (Macina *et al*. 1987). However, we cannot completely rule out the possibility that some parasites would be recognized by several probes, due for exchange to evolutionary convergence of sequences or to horizontal transfer of minicircles among lineages.

kDNA purification was performed as described before (Goncalves *et al*. 1984). kDNA was digested with *Hae*III, electrophoresed on 2% agarose gels, further blotted onto nylon membranes, and hybridized with the random-primed labelled DNA probe. DNA probes NR, CBB and sp 104 corresponding to HVRm of the parasite were prepared and used as already described (Veas *et al*. 1991; Solari *et al*. 2001). *T. cruzi* clone v195 was used here for the first time. The polymerase chain reaction was carried out in order to generate selectively important amounts of the HVRm from the kDNA minicircles. Oligonucleotides were selected from constant regions of the minicircle in order to anneal sites flanking the variable region. An artificial restriction site was

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Table 1. Name of the isolate, geographical origin, date of collection and classification of *Trypanosoma cruzi* isolates by hybridization tests

Name	Host or vector	Region	Probe				Date
			sp104	v195	CBB	NR	
SAP	Human	I	−	−	−	+	1982
FCV	”	I	−	−	−	+	1992
V195	*T. infestans*	I	−	+	+	−	1992
VTV	”	I	−	−	+	+	1992
ACB	Human	I	+	−	−	−	1991
v10P	*T. infestans*	I	+	−	−	−	1982
vMV3	”	I	+	−	−	−	1992
vQUI3	”	I	+	−	−	−	1992
vP1	”	I	+	−	−	−	1982
vFRA2	”	I	−	+	−	−	1992
SPA3	Human	II	−	−	−	+	1990
SPA6	”	II	−	−	−	+	1990
SPA7	”	II	−	−	−	+	1990
SPA9	”	II	−	−	−	+	1990
QUITOR2	*T. infestans*	II	−	−	−	+	1990
v121	”	II	−	−	−	+	1980
v188	”	II	−	−	−	+	1980
CHECAR 1	”	II	−	+	−	−	1990
CHECAR 2	”	II	−	+	−	−	1990
CHECAR 3	”	II	−	+	−	−	1990
QUITOR 4	”	II	−	+	−	−	1990
V124	”	II	+	−	−	−	1990
sp Inca	*M. spinolai*	III	+	−	−	−	1992
GTP	Human	IV	−	−	−	+	1986
ARR	”	IV	−	−	−	+	1986
ATP	”	IV	−	−	−	+	1988
RAM	”	IV	−	−	−	+	1988
FLA	”	IV	−	−	−	+	1986
NT	”	IV	−	−	−	+	1986
WT	”	IV	+	−	−	+	1988
JMJ	”	IV	−	−	−	+	1988
ACG	”	IV	−	−	−	+	1986
LMH	”	IV	−	−	−	+	1986
FSR	”	IV	−	+	−	−	1988
RMS	”	IV	−	+	−	−	1988
CP	”	IV	+	−	−	−	1986
LQ	”	IV	+	−	−	−	1986
v86	*T. infestans*	IV	−	−	−	+	1980
v101	”	IV	−	−	−	+	1980
v1678	”	IV	−	−	−	+	1980
TulC3H	”	IV	−	+	−	−	Not known
TulBalc	”	IV	−	+	−	−	Not known
TulP	”	IV	−	+	−	−	Not known
vOV6	”	IV	−	−	+	−	1980
MCh3	”	IV	−	−	+	−	1980
MxCh53	”	IV	−	−	+	−	1980
CHI22	*T. spinolai*	IV	+	−	−	−	1980
sp104	”	IV	+	−	−	−	1988
sp161	”	IV	+	−	−	−	1988
spCOMB1	”	IV	+	−	−	−	1988
spCOMBr	”	IV	+	−	−	−	1988
PM-MIV	Human	V	−	−	−	+	1992
MCS	”	V	−	−	−	+	1992
RN-GRO	”	V	−	−	−	+	1992
MC-O	”	V	−	−	−	+	1992
spTi5	*M. spinolai*	V	+	−	−	−	1992
spTi9	”	V	+	−	−	−	1992
MF2	Human	RM	−	+	−	−	1992
RFMA	”	RM	−	−	−	+	1992
MF	”	RM	−	−	−	+	1992
HFLA	”	RM	−	−	−	+	1992
CA I	”	Argentina	+	−	−	−	Not known
CL Brener	”	Brazil	−	−	−	−	Not known

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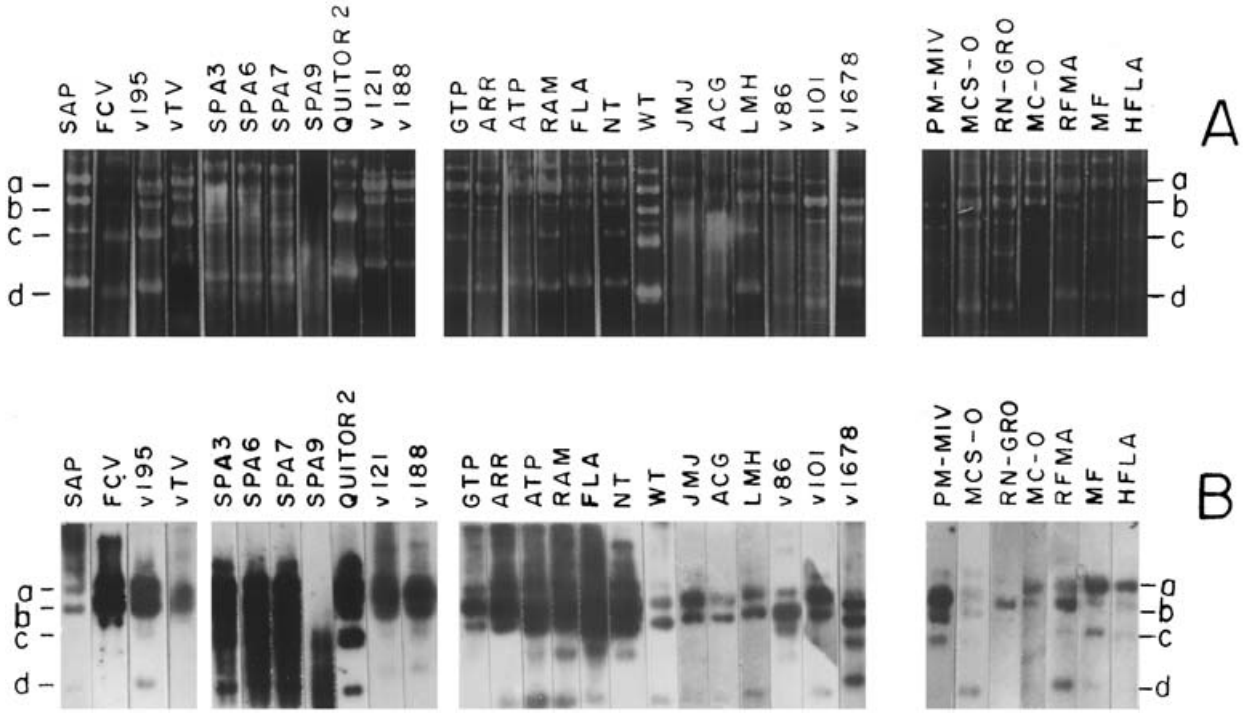

Fig. 1. Detection of *Trypanosoma cruzi* isolates. kDNA from each isolate was digested with *Hae*III and run on 2% agarose gels (A). The blotted gels were hybridized with NR probe (B). The results are from different gels, and hence RFLPs are not identical. a, b, c, and d represent the full size, $\frac{3}{4}$, $\frac{1}{2}$, and $\frac{1}{4}$ minicircle, respectively. The isolate SPA9 gave a RFLP of partially degraded minicircles.

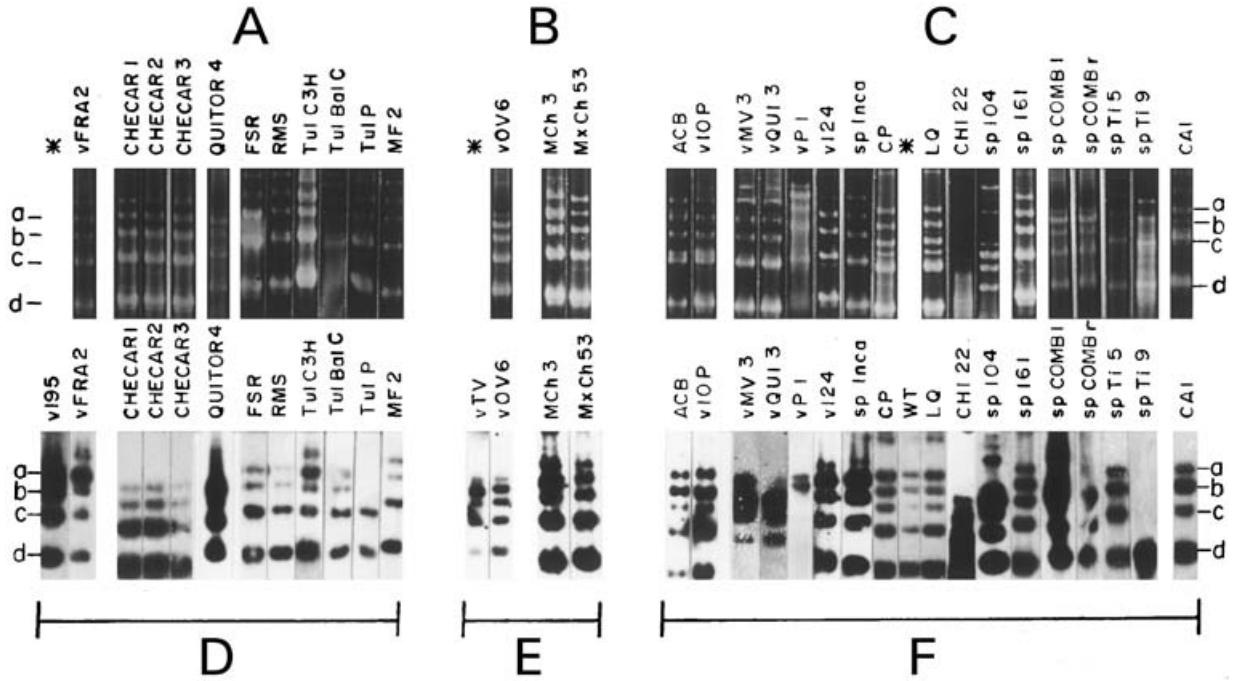

Fig. 2. Detection of *Trypanosoma cruzi* isolates. kDNA from each isolate was digested and electrophoresed as in Fig. 1. Ethidium bromide staining is shown (A, B, and C). The blotted gels were hybridized with v195, CBB, and sp104 probes, respectively (D, E, and F). The molecular weight markers are the same as in Fig. 1. *Represent isolates which cross-reacted with two different probes. The isolates CHI22, and spTi9 gave RFLP with partially degraded minicircles.

introduced close to the 3′ end of each oligo primer, allowing a fast and easy purification of HVRm ready to use as a probe. High stringency hybridization was used: 55 °C in 2 × SSC, washed at 55 °C in 0·1 × SSC. To achieve good signals in an overnight exposure, preparations were hybridized in a solution (10 ml) containing 2×10^6 cpm of ^{32}P-labelled DNA probe (specific activity $50–80 \times 10^6$ cpm/μg DNA),

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or using the alkaline phosphatase chemiluminescent detection method (Amersham, England).

RESULTS

Southern analyses

At the beginning of this work we knew the isoenzyme composition of some isolates (Barnabe *et al*. 2001). Therefore we started the Southern analysis with well-defined HVRm from unique genotypes as probes (NR clone3, CBB clone3, sp104 clone1 and v195 clone1). Figures 1 and 2 show the RFLP and hybridization tests with these probes. One particular *T. cruzi* genotype had peculiar minicircles (Fig. 1). Hybridization with probe NR allowed identification of this genotype since its profile showed mainly bands of 330 bp ($\frac{1}{4}$ minicircles), 1100 bp ($\frac{3}{4}$ minicircles) and 1400 bp (full size minicircles), but rarely showed the one close to 700 bp ($\frac{1}{2}$ minicircles), which was detected in all other isolates studied here (Fig. 2).

Analysis of isolates from different geographical regions of Chile

Parasites found in patients and *T. infestans* of the extreme North (I and II regions) mainly belonged to 3 groups. In this sample the most frequent was the group identified with the v195 probe, followed by the groups detected with the sp104 and NR probes. At least 2 isolates (v195, and vTV) were mixed populations since kDNAs cross-hybridized with 2 of the 4 probes used.

Parasites from the III and IV regions, were from patients, *T. infestans* and the wild vector *M. spinolai* which ranges from 25°S up to central regions of Chile (34°S). The types most frequently found were those hybridizing with the NR probe, followed by the sp104, v195, and CBB probes. Interestingly stocks from *M. spinolai* only hybridized with the sp104 probe. Only 1 isolate corresponding to a mixed population was found with this kind of analysis (isolate WT cross-hybridized with the NR and sp104 probes). Isolates from the central regions of Chile corresponded to the V and Metropolitan Regions, the southern limit of the disease where it is least endemic. Six of the 10 isolates from this region were detectable with NR, followed by stocks detected with the sp104 and v195 probes. Again the stocks which hybridized with the sp104 probe were isolates from *M. spinolai*.

One *T. cruzi* isolate from Argentina was included in this study. CA I hybridized with the sp104 probe, suggesting that this genotype is also frequent in neighbouring countries. Another *T. cruzi* isolate from Brazil (CL Brener), did not hybridize with any probe (not shown).

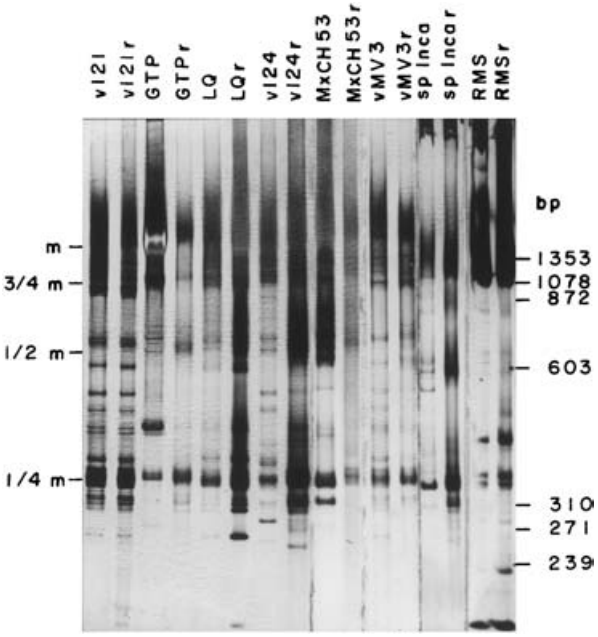

Fig. 3. Schizodemes of *Trypanosoma cruzi* isolates before and after maintainance in the murine model (r). Digestion of kDNA samples with *Eco*RI. Polyacrylamide gradient gel electrophoresis and staining were performed as described in the Materials and Methods section. Molecular size markers are on the right, and the minicircle size on the left margin, respectively.

Mixed populations detected by Southern analysis

Three *T. cruzi* isolates out of 61 studied by hybridization tests were mixed populations with at least 2 genetically unrelated genotypes. Three cases were from the geographical region I (v195 and vTV), and one from the region IV (WT). The latter was studied earlier by dot-blot hybridization tests using total kDNA labelled by nick-translation as probe, with similar results (Solari *et al*. 1991; Sanchez *et al*. 1993).

Schizodeme analysis of stocks

Total kDNA of some stocks was digested with *Eco*RI. The RFLP permits characterization of stocks since the pattern is complex and unique for each stock when electrophoresed in acrylamide gels. Fig. 3 shows an analysis of this kind with a panel of *T. cruzi* populations. Minicircles have restriction endonuclease sites generally located equidistant in 4 highly conserved sequences or constant regions. The restriction fragment length polymorphisms (RFLP) are characteristic for each isolate with DNA fragments close to $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, and the full size of the minicircle.

Eight of the populations studied here were maintained in mice to determine their schizodeme. After the parasite strains were stabilized in mice by several blood passages, each strain was isolated and cultured in liquid medium for further schizodeme analysis

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with *Eco*RI. Results shown in Fig. 3 demonstrate the schizodeme changes in 5 cases after infection in this murine model, suggesting mixed populations in some *T. cruzi* isolates. However, v121, LQ and RMS maintained their schizodeme profile, a strong indication that they are clonal populations.

DISCUSSION

The 4 *T. cruzi* populations used to prepare HVRm probes were cloned and characterized by isoenzyme analysis at 22 variable genetic loci (Barnabe *et al.* 2000). These probes correspond to the major phylogenetic lineages of *T. cruzi*: *T. cruzi* II d contains NR, *T. cruzi* II c contains v195, and *T. cruzi* II b contains CBB; whereas *T. cruzi* I contains sp104.

Single insect vector and human may be simultaneously infected with more than one genotype of *T. cruzi*. We found that it is possible to obtain two very different parasite genotypes in 3 out of the 61 isolates studied by hybridization tests. This represents an underestimation of mixed populations that occurs in nature for at least three reasons. First, parasite growth in culture media may select one parasite from the mixture. Secondly, the HVRm probes used here do not detect individual *T. cruzi* clones but genetically related clones. Thirdly clones present at a lower level may not have been detected in the parasite sample.

Aiming to study further whether *T. cruzi* isolates are mixed populations, we infected mice with 8 *T. cruzi* isolates chosen randomly. Intraspecific variation in *T. cruzi* isolates has long been observed when natural or artificial mixtures of parasites are maintained in experimental models, and when highly discriminative methods, such as schizodeme analysis are used (Deane *et al.* 1984; Carreño *et al.* 1987). Re-isolation after several passages through animals and culture changed the kDNA profile (schizodeme) in 5 of the 8 *T. cruzi* populations tested. In 1 case such as isolate GTP schizodemes before and after passage were radically different, but in the other 4 cases (v124, MxCh53, vMV3, and spInca), the change was minor. This method of high discriminative power indicates the real extent of parasite mixtures in nature. Hybridization tests in the meantime, with these sets of *T. cruzi* isolates and the 4 HVRm probes, detected no changes in the hybridization pattern (not shown).

The geographical distribution of homologous isolates studied by Southern analysis is complex. The isolates detected with the HVRm NR probe were found in all geographical regions in domestic vectors (*T. infestans*) as well as in humans. Out of the 61 isolates studied (not considering 3 Tulahuen strains, CAI and the CL Brener clone), 31 (51%) correspond to isolates which hybridized to the probe derived from genotype clonet 39, which is the most abundant and ubiquitous one in Chile. Another frequently found *T. cruzi* genotype is that detected with the HVRm sp104 probe (17 isolates and 28%). This genotype is detected in the extreme North of Chile (I and II regions) in the domestic *T. infestans* vector, and in the more central regions (III, IV, V and Metropolitan regions) in *M. spinolai*, and in a few humans (Apt *et al.* 1984). This genotype also circulates in *T. infestans* in Perú, but not in patients from southern Perú (Allen, 1984). Interestingly the sp104 probe was detected as homologous to the Argentinean CA I isolate (this study), suggesting this *T. cruzi* genotype is ubiquitous on the eastern side of the Andes. It has also been found in a highly endemic area of Argentina (Macina *et al.* 1987), and seems to be the most pathogenic in this country (Montamat *et al.* 1996, 1999). These isolates hybridized to the probe derived from genotype 19, which corresponds to at least 2 Chilean genotypes of *T. cruzi* determined by molecular karyotyping (Solari *et al.* 1998; Venegas *et al.* 1997). However, the population structure of *T. cruzi* in Bolivia resembles that found in Chile (Barnabe *et al.* 2000). Another less frequent *T. cruzi* genotype circulating in Chile is the one detected by the probe CBB. It has been found in *T. infestans* and humans and corresponds to isolates which hybridized to the probe derived from genotype 32.

An abundant *T. cruzi* genotype found for the first time in Chile is the one detected by the HVRm of v195 probe (9 isolates which represents 15%). This parasite type was previously characterized and typed by molecular karyotyping and schizodeme analysis (Venegas *et al.* 1997). This group of parasites correspond to isolates which hybridized to the probe derived from genotype 36 (Barnabe *et al.* 2000; Barnabe *et al.* 2001), and was found in the extreme North of Chile in *T. infestans*, including 3 Tulahuen strains maintained in mice (Tul C3H, Tul Balc, and Tul P). Finally the Brazilian CL Brener (*T. cruzi* II e) did not hybridize with any probe used here.

It remains to be determined where other genotypes circulating in other areas of America, for example (classical Z 3), and (clonets 40–43) are also present in Chile (Barnabe *et al.* 2000).

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