EXPERIMENTAL PARASITOLOGY **89,** 285-295 (1998)
ARTICLE NO. PR984295

Different Behavior of Two *Trypanosoma cruzi* Major Clones: Transmission and Circulation in Young Bolivian Patients

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Breniere, S. F., Bosseno, M.-F., Telleria, J., Bastrenta, B., Yacsik, N., Noireau, F., Alcazar, J.-L., Barnabe, C., Wincker, P., and Tibayrenc, M. 1998. Different behavior of two *Trypanosoma cruzi* major clones: Transmission and circulation in young Bolivian patients. *Experimental Parasitology* **89,** 285–295. Specificity of two widespread *Trypanosoma cruzi* clonal genotypes or "clonets" (20 and 39) was first analyzed by hybridization with a large set of *T. cruzi* stocks characterized by multigenic study relying on both MLEE and RAPD. Then, these clonets were detected in the blood of Chagasic children from a Bolivian endemic area by a combination of polymerase chain reaction and clonet-specific DNA hybridization. The distribution of these clonets in patients was significantly different from that observed in the vectors of the same area (*Triatoma infestans*). In vectors, clonets 20 and 39 are found with comparable frequencies (0.69 and 0.67, respectively) in contrast with patients, in whom clonet 20 and mixed infections exhibit low frequencies. The Chagasic population can be divided into acute infections and latent infections above the accepted criterion of parasitemia (direct microscopic examination). The results suggest a limited selection in the transmission of the two clonets and a further drastic control of clonet 20 parasitemia by the immune system of children patients.

Index Descriptors and Abbreviations: Trypanosoma cruzi; Triatoma infestans; Chagas disease; clones; PCR; MLEE; RAPD; human transmission.

INTRODUCTION

Trypanosoma cruzi, the agent of Chagas disease, exhibits in nature a high genetic variability, as widely demonstrated by different genetic markers (Miles 1983; Tibayrenc and Ayala 1988; Gibson and Miles 1986; Solari *et al.* 1992; Steindel *et al.* 1993; Tibayrenc *et al.* 1993). The *T. cruzi* populations have a basically clonal population structure (Tibayrenc *et al.* 1986), but genetic exchange may occur in a particular sylvatic cycle (Carrasco *et al.* 1996). The term "clonet" was proposed by Tibayrenc and Ayala (1991) to identify all isolates that appear identical in a clonal species with a given set of genetic markers. The "clones" delimited in *T. cruzi* by either isoenzyme characterization or random primer amplified DNA (RAPD) fingerprinting should rather be considered "clonets." Some clonets appeared widespread and abundant and were named "major clones" (Tibayrenc and Breniere 1988). Lastly, several authors proposed the existence of two major lineages of *T. cruzi* (Tibayrenc 1995; Souto *et al* 1996).

Previously, we designed a molecular clonet identification based upon variable region of minicircle kDNA polymerase chain reaction (PCR) followed by Southern hybridization with clonet-specific kDNA probes of 248 bp (Breniere *et al.* 1992). Probe 20 identified stocks attributed to clonet 20 and certain stocks attributed to clonet 19, whereas probe 39 identified stocks attributable only to clonet 39 [numbering

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0014-4894/98 $25.00

according to Tibayrenc *et al.* (1986)]. The direct identification and characterization of *T. cruzi* clonets circulating in the principal vector of Chagas disease in Bolivia (*Triatoma infestans*) with these probes revealed the actual frequencies of these clonets in the Bolivian domestic cycle and demonstrated an appreciable incidence of mixed infections (Breniere *et al.* 1995; Bosseno *et al.* 1996). A preliminary study also detected the presence of mixed infections in certain patients (Bosseno *et al.* 1995). People living in endemic areas are probably submitted to various reinfections that increase the number of mixed infections detectable in the course of the disease. Nevertheless, the medical consequences of mixed infections in human beings remain to be determined. We applied a modified PCR technique for amplification in blood samples of the variable kDNA minicircles and obtained an optimal sensitivity of this technique compared with conventional serology (Wincker *et al.* 1994).

Chagas disease is a long-persisting chronic infection starting with an acute period lasting 1 or 2 months, in which parasites can be detected in blood by direct microscopic observation. Then the infection becomes latent for a long period in which parasitemia is subpatent. Several factors may take part in the control of parasites, and antibodies seem to play an essential role. Antibodies against *T. cruzi* are able to lyse trypomastigotes and partly block parasite penetration of host cells (Krettli and Brener 1982).

Medical and biological properties of different clonets are poorly documented, although significant biological differences between clonets are observed *in vitro* and *in vivo* (Sanchez *et al.* 1990; Laurent *et al.* 1997; Revollo *et al.* in press).

Given the existence in the Bolivian domestic cycle of two principal clonets (20 and 39), the aim of this work was to improve the direct detection of the major clonets in the blood of young children and to compare their frequencies with those found in *T. infestans* vectors.

MATERIALS AND METHODS

Patients. A population of 270 school-age children from 5 to 10 years old living in the community of Mizque (Cochabamba department), a high endemic Chagas disease area, was simultaneously screened for parasitological and serological diagnosis as well as PCR-based detection. Then 137 of 270 products of amplification, including PCR positive (93) and negative products randomly selected, were Southern hybridized to detect clonets 20 and 39. The parasitemia of these cases was individually controlled by using four buffy coat tubes (BC test, La Fuente *et al.* 1984) for examination. None of these children presented clinical symptoms suggesting a Chagas acute phase.

Vectors. A sample of 128 *Triatoma infestans* was captured from 11 domestic and peridomestic sites in the community of Mizque during the study of the human population. We also captured from various sites 1013 *T. infestans* in four other locations in the Cochabamba department (Ailquile, Quiroga, and Capinota communities and Cochambamba city). The sex of the adults and the nymphal instars was determined for each insect. To detect *T. cruzi* infection, a drop of feces from each insect was mixed with phosphate-buffered saline and microscopically observed for 5 min at an ×400 magnification.

Serological diagnosis. Four assays were used for detection of specific IgG antibodies according to the protocol communicated by Wincker *et al.* (1997): hemagglutination (HEMAVE test, Polychaco, Buenos Aires, Argentine), immunofluorescence (IF), and two enzyme-linked immunosorbant assays (ELISAS) performed in two different laboratories, with the use of crude antigens obtained from epimastigote lysis forms. The diagnosis was based on positivity or negativity of three of four tests.

Polymerase chain reaction diagnosis. The procedure for PCR of fecal samples was according to Breniere *et al.* (1995) with the use of primers CV1 and CV2 (Genset Laboratory, Paris, France), chosen to amplify the hypervariable region of kDNA minicircles (HVRm, Fig. 1). The PCR was applied to microscopically positive feces samples collected in the different localities as well as to negative controls and certain fecal negative microscopic samples.

The PCR procedure in blood was performed in duplicate according to the method described by Wincker *et al.* (1994). The amplification was performed with oligonucleotide primers 121 and 122 (Britto *et al.* 1995), chosen also to amplify the hypervariable region of kDNA minicircles (HVRm). Figure 1 shows the annealing positions of the two sets of primers and their sequences. According to previous experiments, the first set of primers (CV1 and CV2) is useful for direct detection of clones in feces of vectors (Breniere *et al.* 1995), and the second set of primers (121 and 122) appears more sensitive in blood samples (unpublished data). The two sets of primers were also used to amplify the variable regions of minicircles from total DNAs (10 ng) of 45 *T. cruzi* reference stocks characterized by genetic markers

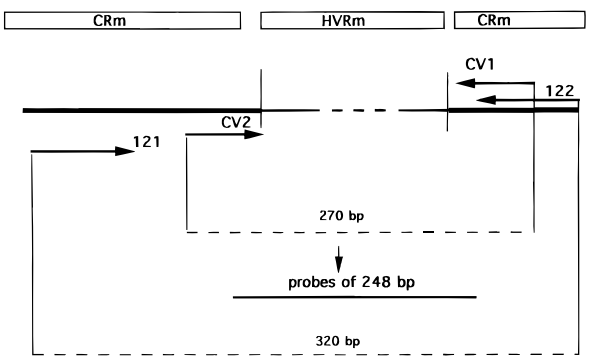

FIG. 1. Diagram of a segment of minicircle of kinetoplast DNA. CRm, conserved region of minicircle; HVRm, hypervariable region of minicircle. Arrows indicate the relative hybridization locations of the CV1 and CV2 set of primers and the 121 and 122 set of primers, which give 270-bp and 320-bp products, respectively. Probes 20 and 39 used in this work (248 bp) are purified from the 270-bp products.

(see later discussion). PCR products were analyzed by electrophoresis on 0.8% agarose gels in TAE × 0.5 and visualized by ethidium bromide staining.

Southern blot. One-tenth of each PCR sample was electrophoresed on an 0.8% agarose in TAE × 0.5 gel that was alkali denatured (0.5 N NaOH, 1.5 M NaCl, twice for 15 min each) and then transferred onto charged nylon membranes (Hybond N+; Amersham, Buckinghamshire, U.K.) by vacuum blotting. For the vacuum transfer, the gel was placed on a prewet membrane, with filter paper followed by a piece of diaper below. This "sandwich" was covered with plastic wrap and placed on a gel dryer (Bio Rad, Paris, France), and vacuum was applied for 10 min without heat. Each membrane included negative controls and two PCR positive controls corresponding to clonets 20 and 39.

Probes. Two clonet-specific probes (numbered 20 and 39) were purified from their respective HVRm DNA fragments produced by the PCR (CV1 and CV2 set of primers) from two *T. cruzi* reference stocks—namely, TPk1, attributed to clonet 39, and S034 cl4, attributed to clonet 20 (Breniere *et al.* 1992). The DNA was digested with restriction endonucleases *Sau* 96 I and *Sca* I (Promega, Madison, WI) to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle.

Labeling and hybridization conditions. Membranes were hybridized by using the enhanced chemiluminescence gene detection system (ECL, Amersham, Buckinghamshire, U.K.). Probes were labeled according to the manufacturer's recommendations for ECL. Briefly, the membranes were incubated at 42°C in hybridization buffer (0.12 ml/cm^2) for 15 min. Each of the purified probes was labeled for 10 min at 37°C. Ten nanograms of labeled probe per milliliter of hybridization buffer was added to the membranes. Hybridization was performed at 42°C overnight in a rotating oven. The membranes were washed twice under highly stringent conditions (6 M urea, 0.1 SSC at 42°C for 20 min) and then twice in 2 × SSC at room temperature for 10 min. Two exposures were performed (1 and 30 min) on Hyperfilm-MP (Amersham, Buckinghamshire, U.K.).

Genetic characterization of reference stocks. Table I summarizes the times of isolation, places, and hosts of the 45 stocks. Among them, 30 were previously characterized by 15–22 isoenzyme loci (Tibayrenc and Ayala, 1988; Breniere *et al.* 1989; Breniere *et al.* 1991; Lewicka *et al.* 1995) and were selected to give a reliable picture of genetic diversity within *T. cruzi*. To diversify the host and geographical origin of the sample, the others stocks were selected. Stocks were sampled from nine different countries (Bolivia, Brazil, Chile, Colombia, French Guyana, Honduras, Mexico, the United States, and Venezuela) and were isolated from various vectors, human and sylvatic mammals. Parasites were bulk-cultured in LIT medium and harvested by centrifugation. Genetic characterization was performed with a total of 22 isoenzyme loci and by RAPD fingerprinting with the use of seven 10-mer primers. Multilocus enzyme electrophoresis (MLEE) was carried out on cellulose acetate plates according to Ben Abderrazak *et al.* (1993). The following 20 enzyme systems were used: aconitase (ACON, EC 4.2.1.3), alanine aminotransferase (ALAT, EC 2.6.1.2), diaphorase (DIA, EC 1.6.*.*), glyceraldehyde-3-phosphate dehydrogenase (GAPD, EC 1.2.1.12), glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), glucose phosphate isomerase (GPI, EC 5.3.1.9), glutamate dehydrogenase NAD$^+$ (GDH NAD$^+$, EC 1. 4.1.2), glutamate dehydrogenase NADP$^+$ (GDH NADP$^+$, EC 1.4.1.4), isocitrate dehydrogenase (IDH, EC 1.1.1.42), leucine aminopeptidase (LAP, EC 3.4.11. or 13.*.), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), mannose phosphate isomerase (MPI, EC 5.3.1.8), nucleoside hydrolase (inosine) (NHi, EC 2.4.2.*.), peptidases (substrates: L-leucylleucineleucine and L-leucyl-L-alanine) (PEP, EC 3.4.11 or 13.*), 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44), phosphoglucomutase (PGM, EC 2.7.5.1.), and superoxide dismutase (SOD, EC 1.15.1.1). For the RAPD analysis, seven 10-mer primers, A1, A2, A4, A7, A9, A10, and A19 (Appligene, Illkirch, France), were used, and conditions were as published in Williams *et al.* (1990) and recommended by Boehringer (Mannhein, France) supplier of the Taq DNA polymerase. Briefly, amplification reactions were performed in a volume of 60 μl containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatine, 10 μM each of dNTP, 0.2 μM primer, 10 ng of genomic DNA, and 0.6 U of Taq DNA polymerase. After mixing, the tubes were placed in a PTC 100, MJ Rechearch Inc. thermal cycler for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. A final extension was performed at 72°C for 7 min. Each protocol included controls without DNA template. Amplification products were analyzed by electrophoresis in 1.6% agarose gels and detected by staining with ethidium bromide. The conditions were optimized to obtain reproducible patterns.

Data analysis. Each RAPD reproducible gel band was coded with a number, starting with 1 for the slowest band (largest DNA fragment). Each stock was thus represented by a set of numbers for each primer. A matrix of presence and absence of each band was deduced from these data for all stocks. The same method was used for the generation of MLEE matrix.

Variability among stocks was estimated by Jaccard's phenetic distances for both MLEE and RAPD (Jaccard, 1908; Tibayrenc *et al.* 1993). The correlation between the two sets of similarity index (Jaccard's distances) was estimated by the Mantel *t*-test (Mantel, 1967).

Genetic variability among stocks was also estimated by patristic distances calculated from one matrix (presence or absence) joining RAPD and MLEE matrixes. Phylogenetic relationships among the stocks were visualized by a minimum-length unrooted Wagner network (Farris 1970; Felsenstein 1978). The support of each node was tested by a bootstrap analysis (Felsenstein 1985).

Comparison of the clonet frequencies were performed by the chi^2 statistical test.

RESULTS

Overall phylogenetic diversity. MLEE and RAPD data gave congruent results, shown by similar topologies of the two dendrograms obtained from Jaccard's distances generated by each method (data not shown). Moreover, a significant correlation between distances was evidenced by the Mantel correlation test ($P < 10^{-4}$). Practically, this means that stocks that appear to be closely related by the use of isoenzymes tend statistically to be closely related when RAPD fingerprinting is used, and conversely. The minimum-length unrooted Wagner network constructed from a unique matrix plotting MLEE and RAPD data (29 loci) is presented in Fig. 2. The stocks are clustered in two groups genetically distant—namely, 1 and 2—except stocks A0087 and A0083 isolated from sylvatic mammals, the taxonomic identity of which remains to be determined. Bootstrap analysis shows

TABLE I

Geographic Origin, Code Number, Date of Collection, and Host of the 45 *Trypanosoma cruzi* Stocks Studied by Isoenzyme and RAPD Genetic Markers

Stock	Host	Locality	Date	MLEE**
Cutia cl1*	*Dasylprocta aguti*	Esperito Santo, Colatina, Brazil		1
Cuica cl1*	*Philander opossum*	Sao Paulo, Riberao Preto, Brazil	nk	1
SO34 cl4*	*Triatoma infestans*	Potosi, Toropalca, Bolivia	7/86	3
Gamba cl1*	*Didelphis azarae*	Sao Paulo, Riberao Preto, Brazil	nk	1
P11 cl3*	Human	Cochabamba, Cochabamba, Bolivia	2/80	2
Esquilo cl1*	*Sciurus aestuans ingrami*	Sao Paulo, Franca, Brazil	nk	1
SP104 cl1*	*Triatoma spinolai*	IVa region, Combarlala, Chile	nk	ND
P209 cl1*	Human	Chuquisaca, Sucre, Bolivia	9/83	2
13379 cl7*	Human	Santa-Cruz, Santa-Cruz, Bolivia	79	1
EV 13-C	*Rodnius prolixus*	Meta, Calaguala, Colombia	81	ND
RTD	*Didelphis marsupialis*	Tolima, Coyaima, Colombia	nk	ND
EP222	*Rodnius prolixus*	Casanare, Cravo Sur, Colombia	79	ND
X10 cl1 Z1*	Human	Para, Belem, Brazil	nk	1
Florida	*Triatoma sanguisuga*	Gainesville, FL, U.S.A.	nk	ND
Tehuantepec cl2*	Triatominae	—,—,Mexico	38	1
Davis	*Triatoma dimidiata*	—, Tegucigalpa, Honduras	83	ND
OPS21 cl1*	Human	Cojedes, Macuayas, Venezuela	6/77	1
OPS4	*Didelphis marsupialis*	Carabobo, El Yagual, Venezuela	12/76	1
Opossum 70	*Didelphis marsupialis*	New Orleans, Crown Point, U.S.A.	70	ND
A0105	*Didelphis marsupialis*	—, Cacao, French Guyana	4/83	1,4
31R16	Human	Santa-Cruz, Santa-Cruz, Bolivia	83	1
NS	Human	Sucre, Galeras, Colombia	nk	ND
A0269	*Philander opossum*	—, Cacao, French Guyana	86	4
A0268	*Philander opossum*	—, Cacao, French Guyana	86	4
CBB cl3*	Human	IVa region, Tulahuen, Chile	nk	3
MAS1	Human	Brazilia, —, Brazil	nk	ND
IVV	Human	IVa region, —, Chile	nk	ND
TU18 cl2*	*Triatoma infestans*	Potosi, —, Bolivia	nk	ND
MVB	Human	IVa region, —, Chile	nk	ND
Esmeraldo cl3 Z2*	Human	Bahia, Sao Felipe, Brazil	nk	1
NT	Human	IVa region, Chillepin, Chile	nk	ND
SO3 cl5*	*Triatoma infestans*	Potosi, Otavi, Bolivia	6/86	3
MN cl2*	Human	IVa region,—, Chile	nk	3
SC43 cl1*	*Triatoma infestans*	Santa-Cruz, Santa-Cruz, Bolivia	5/81	1
86-1	not known	Santa-Cruz, Santa-Cruz, Bolivia	nk	ND
Bug 2149 cl10*	*Triatoma infestans*	Rio Grande do Sul, —, Brazil	nk	1
NR cl3*	Human	IIIa region, Salvador, Chile	nk	3
TPK1	*Triatoma infestans*	La Paz, Khala Khala, Bolivia	10/86	3
Bug 2148 cl1*	*Triatoma infestans*	Rio Grande do Sul, —, Brazil	nk	ND
Tulahuen FKIIA cl2*	Human	IVa region, Tulahuen, Chile	nk	1
M5631 cl5*	*Didelphis novemcinctus*	Para, Selvaterra, Brazil	nk	1
M6241 cl6*	Human	Para, Belem, Brazil	nk	1
Can III cl1 Z3*	Human	Para, Belem, Brazil	nk	1
A0083	*Didelphis marsupialis*	—, Montsinery, French Guyana	82	4
A0087	*Didelphis marsupialis*	—, Montsinery, French Guyana	82	4

*Laboratory cloned stocks

**Previous isoenzyme characterization for 15–20 gene loci, 1, Tibayrenc and Ayala (1988); 2, Breniere *et al.* (1989); 3, Breniere *et al.* (1991); 4, Lewicka *et al.* (1995), ND, not done, nk, not known.

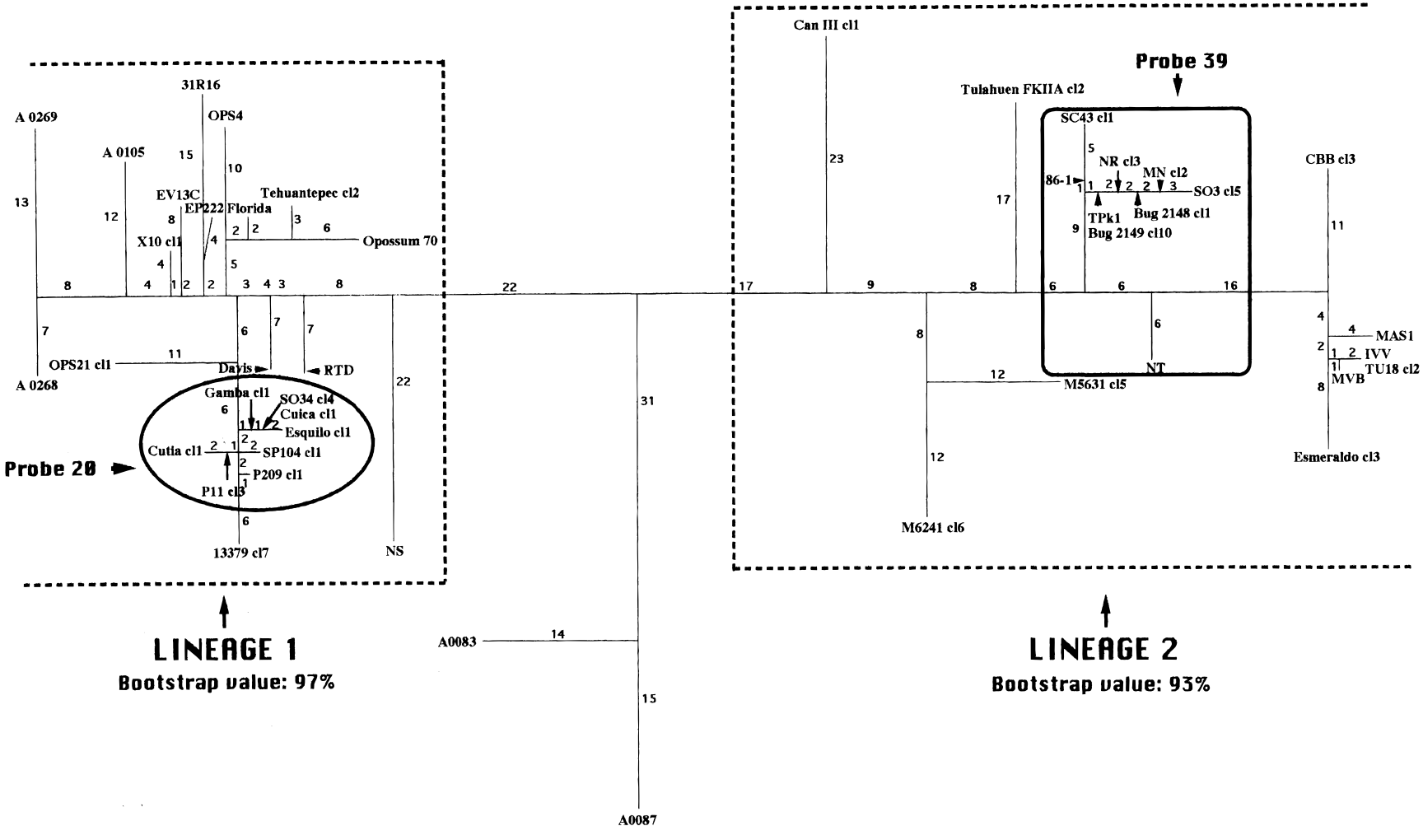

FIG. 2. A minimum-length unrooted Wagner network of 437 steps linking 45 stocks of *Trypanosoma cruzi* analyzed by MLEE and RAPD together. The name of each stock is given at each terminal point branch. The numbers along the branches are the patristic (evolutionary) steps. The circles indicate the groups of stocks recognized by probes 20 and 39, respectively.

that groups 1 and 2 occurred 97 and 93 times out of 100 replicates, respectively. This result supports the existence of two major phylogenetic lineages, as previously proposed (Tibayrenc, 1995). Nevertheless, each lineage is composed of stocks presenting high diversity. Bootstrap analysis shows that, within each lineage, some stocks are gathered together with a high probability. It is worth noting that, in lineage 1, nine stocks (SP104 cl1, Cutia cl1, P11 cl3, 13379 cl7, P209 cl1, Cuica cl1, Esquilo cl1, SO34 cl3, and Gamba cl1) are truly gathered; this group occurred 82 times out of 100 replicates. In lineage 2, we observed six stocks (IVV, TU18 cl2, MVB, Esmeraldo cl3, MAS1, and CBB cl3) and eight stocks (TPK1, Bug 2149 cl10, NR cl3, SO3 cl5, MN cl2, Bug2148 cl1, 86-1, and SC43 cl1) clustered with probabilities of 92% and 89%, respectively.

Specificity of probes 20 and 39. The hybridization patterns of the PCR products of DNA reference stocks generated by each set of primers (CV1 and CV2, 121 and 122) were identical. Thus, any of these primers can be used for direct detection of clonets 20 and 39 in biological samples.

For either probe 20 or probe 39, each hybridization pattern was restricted to a limited number of stocks, and we did not observe any cross-reaction between the probes (Fig. 2). Except for stock 13379 c17, probe 20 hybridized all closely related stocks, which fall into a specific cluster, as evidenced by the Wagner network and the bootstrap analysis (Fig. 2). Similarly, probe 39 hybridized stocks gathered with a high probability and the NT outside group stock.

Identification of clonets 20 and 39 in vectors. All investigated areas are situated in Cochabamba department, where *T. infestans* is well established in domestic and peridomestic sites. The infection rates of the triatomines determined by microscopic observation were 42.6% for the entire population and were even high (30.2%) in the urban area of Cochabamba city (Table II). Hybridization results obtained after transfer of the PCR positive products of fecal samples onto membranes are summarized in Table II. All positive PCR samples showed a major band of 270 bp previously demonstrated to be specific for the *T. cruzi* species (Veas *et al.* 1991). Three different hybridization patterns were observed: (1) samples positive with either probe 20 or probe 39; (2) samples positive with both probes (mixed infections); and (3) samples negative with both probes. The control samples were all PCR and hybridization negative as expected.

In the Mizque community, the frequencies of clonets 20 and 39 were 0.69 and 0.67, respectively, of which 43.1% were mixed infections. The vast majority of the insects appeared to be infected by clonet 20 or 39 or both. However, four samples (7.8%) were not recognized by either of the two probes, suggestive of the presence of other clonal genotypes, whose taxonomic identity remains to be determined.

In the other localities of the Cochabamba department, we also observed similar frequencies of clonets 20 and 39, except in the Capinota region, where clonet 20 appeared to be more frequent than clonet 39. Mixed infections also were abundant, ranging from 31.1% to 85.7% in the locality of Quiroga. In these other locations, 7.4% of the insects were probably infected by other clones (samples that are not recognized by probe 20 or 39). This percentage is similar to that found in the locality of Mizque. In the whole population, clonet distribution was analyzed according to sex and nymphal instars by the chi^2 test (Table III). The results suggest an absence of clone selection according to sex or nymphal instars of the insect (Yates's corrected $\chi^2 = 15.68$, $df = 9$, $P > 0.05$).

Identification of clonets 20 and 39 in patients. Among the 270 schoolchildren surveyed, 110 presented a positive IgG serology (40.7%); 1 gave a doubtful result (two positive tests and two negative tests). In the whole population, 12 (4.4%) children presented a positive direct parasitological diagnosis (buffy coat) and were considered recently infected (acute cases). One hundred six children were PCR (+) (39.2%), and the agreement between the two techniques (PCR and IgG serology) was 95.5%. The patient with doubtful results presented a PCR (+). Table IV presents the hybridization results of 137 PCR products including the majority of PCR (+) and 44 randomly selected PCR (−) products as hybridization control. We routinely prepared one negative control (a blood lysate from a normal person) and one positive control (a blood lysate from a positive Chagasic patient) for every five samples tested. Each DNA extraction was performed in duplicate for each patient, and PCR was performed independently on these two preparations; a typical result for a series of PCR tests is shown in Fig. 3. As for vector feces hybridization, we observed three different hybridization patterns (Fig. 4) that correspond to the identification of either clonet 20 or clonet 39 or both. The cases of PCR (+) with absence of recognition of amplified products by any of the two probes probably correspond to other clones genetically unrelated to clonets 20 and 39. Among the 44 PCR (−) products, 41 showed no hybridization, as expected (Table IV). Nevertheless, 3 of these PCR (−) samples were hybridized by probe 39. Two of them corresponded to patients with positive IgG serology (Chagasic patients). The hybridization step can be sensitive enough to detect amplified products not detectable by ethidium bromide staining. The third patient had a negative IgG serology and a contamination problem can be suspected. Among the 93 PCR (+) products, a large majority were hybridized by

TABLE II
Identification of Clonets 20 and 39 of *Trypanosoma cruzi* by Specific Probes in PCR-Amplified Feces of Vectors

Location	Microscopic Results		Clonet Identification, No. (%)				
	No.	% of Positive	No.	20 only	39 only	20 and 39	Neither 20 nor 39
Mizque	128	74 (57.8)	51	13 (25.5)	12 (23.6)	22 (43.1)	4 (7.8)
Ailquile	205	72 (35.1)	38	10 (26.3)	6 (15.8)	21 (55.3)	1 (2.6)
Quiroga	57	31 (54.4)	14	0 (0.0)	2 (14.3)	12 (85.7)	0 (0.0)
Capinota region	506	235 (46.4)	22	10 (45.5)	2 (9.1)	8 (36.3)	2 (9.1)
Cochabamba	245	74 (30.2)	74	23 (31.1)	20 (27.0)	23 (31.1)	8 (10.8)
Total	1141	486 (42.6)	199	56 (28.1)	42 (21.1)	86 (43.2)	15 (7.6)

probe 39 (85%). In only one patient did we identify clonet 20 alone, and 18.3% of the patients presented a mixture of the two clonets. Nine of 93 patients with PCR (+) presented a positive buffy coat (acute cases). Clonet distribution in this group therefore appears different from the clonet distribution in the group of patients with negative buffy coat (84 latent infections, Tables IV and V). The patients in the acute phase present a higher frequency of clonet 20 and of mixed infections (Yates's corrected $\chi^2 = 12.8$ and $= 14.0$, respectively, with $df = 1$ and $P < 0.001$).

Comparison of the clonet distribution in vectors and patients. Table V summarizes the clonet identification in the vectors and in the patients of Mizque according to the stage of infection. The clonet distribution is radically different in vectors and in the entire population of patients ($\chi^2 = 41.8$, $df = 3$, $P < 0.0001$). Clonet 20, which is abundant in vectors (frequency = 0.69), is little represented in the blood circulation of patients (frequency = 0.19), $\chi^2 = 35.4$, $df = 1$, $P < 0.001$. The number of mixed infections falls proportionally in patients, $\chi^2 = 10.8$, $df = 1$, $P < 0.01$. A significant increase in the percentage of patients infected by clonet 39 is observed ($\chi^2 = 6.8$, $df = 1$, $P < 0.02$). In contrast, when we compared the distribution of the clonets in vectors and in the patients presenting a positive buffy coat (acute cases), we did not observe a significant difference between the respective infection rates by clonet 20 ($\chi^2 = 0.01$, $df = 1$, $P > 0.05$). The clonet distribution in the group of latent infections is similar to that obtained for the entire population.

TABLE III
Instar Distribution of Clonets 20 and 39 in *T. infestans*.

	Clonet Identification No. (%)			
	Adults		Nymphs	
	Male	Female	Instars 1–4	Instar 5
20 only	14 (25.5)	14 (24.1)	18 (29.5)	5 (33.3)
39 only	8 (14.5)	13 (22.4)	15 (24.6)	6 (40.0)
20 and 39	30 (54.5)	29 (50.0)	20 (32.8)	4 (26.7)
Neither 20 nor 39	3 (5.5)	2 (3.5)	8 (13.1)	0 (0.0)
Total	55	58	61	15

Note. $\chi^2 = 15.68$; $df = 9$; $P > 0.05$.

DISCUSSION

Phylogenetic specificity of probes 20 and 39. The present results make it possible to have a better idea of the specificity of probes 20 and 39. As supported by the phylogenetic analysis with MLEE and RAPD on representative set of stocks, the

TABLE IV
Identification of *Trypanosoma cruzi* Clonets 20 and 39 by Specific Probes in PCR Amplified Blood Samples of Mizque Infantile Population

PCR	Buffy Coat*	Clonet Identification, No. (%)**				
		No.	20 only	39 only	20 and 39	Absence of Recognition
+	+	9	0 (0.0)	3 (33.3)	6 (66.7)	0 (0.0)
$n = 93$	−	84	1 (1.2)	59 (70.2)	11 (13.1)	13 (15.5)
−	+	1	0 (—)	0 (—)	0 (—)	1 (—)
$n = 44$	−	43	0 (0.0)	3 (6.9)	0 (0.0)	40 (93.1)

*Buffy coat, direct parasitological diagnosis by microscopical examination of four capillary interphases.

**(—), not calculated (sample too small).

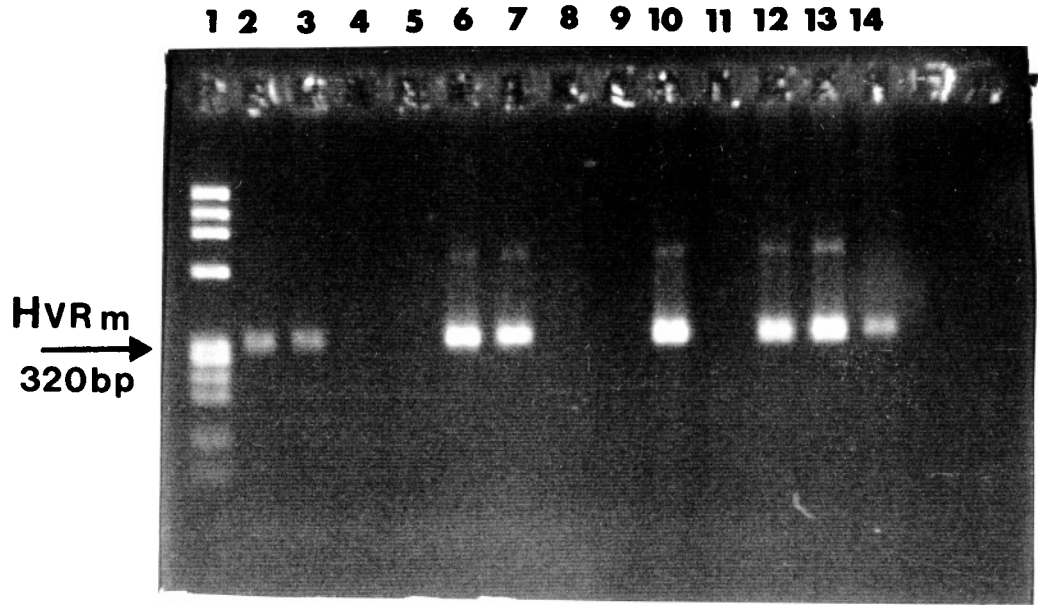

FIG. 3. Typical PCR-based diagnosis by using samples from five children of Mizque, processed and amplified in duplicate and revealed by ethidium bromide staining after migration on 2% agarose gel. Lane 1, *Hae* III digested OX 174 DNA. Lanes 8 and 9, negative control sample (from a non-Chagasic person processed together with the five children). Lanes 14, positive control (confirmed Chagasic patient). Lanes 2 and 3, 6 and 7, and 10 through 13, four different patients with a positive PCR-based diagnosis. Lanes 4 and 5, one patient with a negative PCR-based diagnosis.

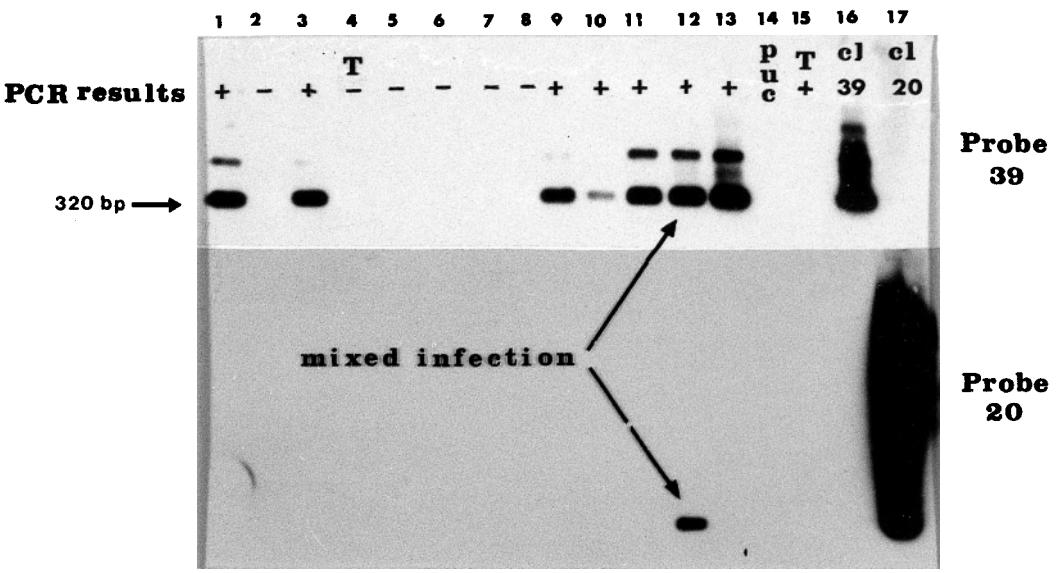

FIG. 4. Hybridization patterns of PCR products from 12 different children with clone-specific probes 20 (below) and 39 (above). Lane 4, negative control (from a non-Chagasic person). Lane 15, positive control (confirmed Chagasic patient with positive PCR); this patient is from Brazil and is not infected by clone 20 or 39. Lanes 16 and 17, positive controls, DNAs of reference *T. cruzi* stocks pertaining to natural clones 20 and 39, respectively. Lanes 1, 3, 9 through 11, and 13, patients presenting a positive PCR; the products are recognized only by probe 39. Lane 12, patient presenting a positive PCR; the products are recognized by both probe 20 and probe 39 (mixed infection). Lanes 2 and 5 through 8, patients presenting a negative PCR. Lane 14, DNA size marker *Rsa* I digested by Puc 19.

TABLE V
Comparison of the Clonet Distribution in Vectors and Patients from the Mizque Locality

	Clonets Identification in			
	Vectors	Entire population of chagasic patients*	Acute cases	Latent infections
20 only	13 (25.5%)	1 (1.1%)	0 (—)	1 (1.2%)
39 only	12 (23.6%)	64 (67.4%)	3 (33.3%)	61 (70.9%)
20 + 39	22 (43.1%)	17 (17.8%)	6 (66.7%)	11 (12.8%)
Neither 20 nor 39	4 (7.8%)	13 (13.7%)	0 (—)	13 (15.1%)
Total	51	95	9	86
Frequency of clonet 20	0.69	0.19	0.67	0.14
Frequency of clonet 39	0.67	0.85	1.00	0.84

*Includes the patients with positive PCR and the two patients with negative PCR but positive hybridization associated with positive IgG serology.

two probes specifically label delimited clusters that probably correspond to phylogenetic clades. Probes 20 and 39 should be equated to synapomorphic characters (derived characters that are common to all members of a given clade, and only to them). The two clonets appear to exhibit radically distinct biological properties and tend to behave as distinct taxa (Laurent *et al.* 1997; Revollo, 1995). Interestingly, other major clonets (32 and 33, see Tibayrenc *et al.* 1986) were also present in the set of stocks analyzed by MLEE and RAPD. They were not recognized by probes 20 and 39 (Veas *et al.* 1991), which corroborates the strict specificity of these probes. Agreement between MLEE and RAPD results, on the one hand, and kDNA probe results, on the other hand, confirms overall parallel evolution between nuclear and kinetoplast genomes (Tibayrenc and Ayala, 1988). These results do not favor the hypothesis that kDNA is similar to bacterial plasmids. Moreover, parallel results between nuclear (MLEE, RAPD) and kDNA (probes 20 and 39) markers indicate a strong linkage disequilibrium between the two sets of markers, which is additional evidence for clonal evolution in *T. cruzi* (Tibayrenc *et al.* 1986; Tibayrenc 1995).

Compared frequencies of clonets 20 and 39. *Triatoma infestans* is the main vector in the domestic cycle in Bolivia, where it makes a steady active transmission of Chagas disease in various departments. In Cochabamba department, clonets 20 and 39 are always found frequently, as well as in the La Paz department, where we reported 65.8% and 56.9% of triatomines infected by clonets 20 and 39, respectively (Bosseno *et al.* 1996). Both clonets appear to be widespread over large geographical distances, because the localities surveyed in the La Paz and Cochabamba departments are about 400 to 600 km apart.

We observed a high discrepancy in the clonet distribution in vectors and in patients of the Mizque community. If the null hypothesis of independent transmission and a lack of interaction between the two groups of clonets are assumed, similar distributions of the clonets in vectors and patients are expected. Several hypotheses could explain this discrepancy.

(1) *Temporal fluctuation of clonet frequencies in vectors.* The clonets were detected in vectors and patients simultaneously. Chagas disease is a long-lasting infection, and a majority of the patients were probably infected several years before the study. A different clonet distribution in vectors in the past could explain the discrepancy currently observed. Twelve years ago, Tibayrenc *et al.* (1986) reported in various locations of Bolivia and in Cochabamba department comparable high frequencies of the two clonets in vectors. This result and the lasting presence of the two clonets in vectors over large geographical distances support the absence of temporal fluctuations in the clonet frequencies in vectors.

(2) *Selection of a specific clonet in humans.* The selection of one specific clonet in patients should be explained by differences of infectivity or parasitemia control by the immune system. To analyze the infectivity of the clonets, it is necessary to compare the clonet distributions in vectors and in patients with recent infection. If the absence of an established immune response and the low probability of multiinfection are assumed, a similar distribution in these patients and vectors is expected. In the current sample, the distribution of the clonets in patients presenting a positive parasitemia, patients that should correspond to those with most recent infection, was not significantly different from that of the vectors. Moreover, in a previous study, we observed a similar result; we reported the presence of clonets 20 and 39 in children living in Cochabamba city (Bosseno *et al.* 1995). These patients, between a few months of age and 2

years old, had a recent infection (acute cases), as confirmed by a positive parasitemia. Among these 15 patients, 3 were infected by clonet 20 only, 7 were infected by clonet 39 only, 4 were infected by the two clonets together, and 1 was not infected by either of the two clonets. The distribution of the clonets in this second group of children is similar to that found in the vectors of Cochabamba city ($\chi^2 = 2.4$, $df = 3$, $P > 0.05$, see Table II). The results of the two samples of patients in acute phase favor the hypothesis of similar transmission behavior of the two clonets. Consequently, the selection should occur later in the infection.

Previously, 89 stocks isolated by xenodiagnosis from adult Bolivian patients (chronic infection) were characterized by isoenzyme analysis after culture: 42% and 50% of them were infected by clonets 20 and 39, respectively (Breniere *et al.* 1989); among them, the patients from the Cochabamba department presented similar frequencies of the two clonets. The discrepancy between these results and the current observation in children of clonet 39 predominance can be explained by two recent experimental works. Stocks belonging to clonet 20 present a higher multiplication rate in *T. infestans* (Pinto *et al.* in press). Moreover, the growth velocity of clonet 20 is superior to that of stocks belonging to clonet 39 (Laurent *et al.* 1997). Consequently, the indirect characterization of patient stocks presents a significant bias, and a direct characterization by PCR is more suitable.

Epidemiological model. We proposed an epidemiological model that might explain the major detection of clonet 39 and the low frequency of clonet 20 in patients, in view of the maintenance of the two clonets in vectors.

(1) *Human multiinfecting contacts.* Because clonet 20 and 39 are dominant and frequently associated together in vectors, the rate of human mixed infections should increase rapidly after various infecting contacts. The patients infected by only one of the two clonets should be the exception. As observed, inferring the lack of clonet 20 detection, only clonet 39 should be detected in most patients. This model of multiple human infections is mostly according to the observed clonet frequencies.

(2) *Synanthropic mammals–vector cycle.* In a previous study, we analyzed the proportion of clonets 20 and 39 and of mixed infections in vectors from various Bolivian areas (Bosseno *et al.* 1996), showing the absence of natural selection during the cycle. These data are in apparent conflict with the present work, which supports a selection of clonet 39 by the human host. Consequently, a restricted human–vector cycle should tend to the disappearance of clonet 20 in vectors. The synanthropic animals—dogs, cats, and rodents—should play an important role for the maintenance of the two clonets. Each mammal host could present different susceptibility to the clonets. In an experimental mouse model, authors showed the high infectivity and parasitemia of stocks belonging to clonet 20, in contrast with clonet 39 (Sanchez *et al.* 1990; Laurent, 1997).

Therefore, the intervention of various hosts in the domestic cycle could explain the persistence of clonet diversity in vectors.

ACKNOWLEDGMENT

This work was supported by a grant from the World Health Organization Special Program for Research and Training in Tropical Diseases (No. 910273).

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Received 6 May 1997; accepted with revision 23 February 1998