DIRECT IDENTIFICATION OF *TRYPANOSOMA CRUZI* NATURAL CLONES IN VECTORS AND MAMMALIAN HOSTS BY POLYMERASE CHAIN REACTION AMPLIFICATION

S. F. BRENIERE, M. F. BOSSENO, S. REVOLLO,
M. T. RIVERA, Y. CARLIER, AND M. TIBAYRENC

Made in United States of America
Reprinted from THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE
Vol. 46, No. 3, March 1992

Am. J. Trop. Med. Hyg., 46(3), 1992, pp. 335–341 (91-018)

DIRECT IDENTIFICATION OF *TRYPANOSOMA CRUZI* NATURAL CLONES IN VECTORS AND MAMMALIAN HOSTS BY POLYMERASE CHAIN REACTION AMPLIFICATION

S. F. BRENIERE, M. F. BOSSENO, S. REVOLLO, M. T. RIVERA, Y. CARLIER, AND M. TIBAYRENC

Laboratoire de Genetique des Parasites et des Vecteurs, ORSTOM, Montpellier, France; IBBA, Departamento de Parasitologia, La Paz, Bolivia; Laboratoire de Parasitologie, Universite Libre de Bruxelles, Bruxelles, Belgium

Abstract. The polymerase chain reaction was used to amplify the highly variable region of the kinetoplast minicircle of *Trypanosoma cruzi* directly in biological samples (feces of infected Triatomine bugs, blood samples of experimentally infected mice, and artificially infected human blood samples). Hybridization of the amplified DNAs with reference stocks representing different genotypes (natural clones) enabled us to characterize the stocks infecting the biological samples under study. The main interest of this new approach is the diagnosis of *T. cruzi* infection and simultaneous direct identification of the different natural clones circulating in vectors and mammalian blood without isolation of the stocks. The suitability of this technique for epidemiologic studies is also discussed.

Natural populations of *Trypanosoma cruzi,* the agent of Chagas' disease, have a basic clonal structure, which has been analyzed by extensive population genetic studies.[1–3] The previously described zymodemes and schizodemes can be equated to natural clones, as shown by isozyme or restriction fragment length polymorphism analyses, respectively.[4, 5] These natural clones should be considered as useful taxonomic units in any applied study.[6] Among the large number of different natural clones isolated, some appear to be ubiquitous and are frequently encountered in endemic areas. These dominant genotypes, which are called major clones, deserve special attention.[7] Nevertheless, the biological and medical peculiarities of these various clones remain poorly defined. At present, identification of these natural clones requires 1) isolation of the parasites from vectors or mammalian hosts, 2) culture amplification, and 3) multilocus characterization by isoenzyme techniques. Isolation of *T. cruzi* stocks from vectors is relatively simple, but it lacks sensitivity when attempted with chronically infected patients and mammalian hosts because of their low level of parasitemia.[8, 9] Selection pressure may occur with natural clones through isolation from culture or mice.[10, 11] Moreover, multilocus isozyme characterization is both time-consuming and expensive. For these reasons, fast, specific, and sensitive labeling of *T. cruzi* major clones is needed for field or experimental studies directed toward defining clinical characteristics.

Previous work has shown that the hypervariable region of the *T. cruzi* kinetoplast DNA (kDNA) minicircle (HVRm) exhibits sequences that are specific for the natural clones identified by our multilocus isozyme characterization. This has led to the development of DNA probes for some *T. cruzi* major clones.[12] These results are based upon and favor the hypothesis of linked evolution between nuclear DNA (labeled by isozyme analysis) and kinetoplast DNA.[13] Thus, the HVRm of kDNA can be considered a clone marker. In this report, we propose direct characterization of *T. cruzi* natural clones isolated from infected feces and blood using clone-specific HVRm DNA sequences as new markers that are easily obtained by the PCR technique.

MATERIALS AND METHODS

Samples

Four different types of samples were investigated: 1) triatomine feces, 2) blood from adult patients, 3) artificially-infected human blood, and 4) blood samples from experimentally infected mice. All triatomines *(Triatoma infestans)* were isolated in Bolivia. Some of them were collected

in endemic areas, while others originated from xenodiagnosis procedures performed on patients. Microscopic examinations for positive infection were performed (by checking at least 60 microscopic fields at 40× magnification) before recovering the fecal samples. All patient blood samples were collected in Bolivia. Some of the patients exhibited positive serology by both immunofluorescence analysis and enzyme-linked immunosorbent assay, while other patients had negative results for these tests.[14]

BALB/c mice were infected with 100–1,000 trypomastigote blood forms of a laboratory cloned stock of *T. cruzi* that was previously used in an experimental mouse model at the Laboratory of Parasitology at the University of Brussels. This clone is genetically related to clone 43, as shown by isozyme analysis. (In the present work, all clones are numbered according to the recommendations of a previous report.[1] The term clone denotes a natural clone of *T. cruzi* identified by population genetic analysis rather than a laboratory clone.) Parasitemia levels in infected mice were evaluated by examination of blood samples.[15]

Processing of fecal and blood samples for PCR analysis

Samples of triatomine feces (10–20 μl) were individually collected in microtubes, diluted with 200 μl of distilled water, boiled for 10 min, and centrifuged at 8,000 *g* for 10 min. Mouse blood samples (100 μl) were collected by intracardiac puncture, diluted with 100 μl of distilled water, boiled for 10 min, and centrifuged at 8,000 *g* for 10 min. Samples (100 μl) of artificially infected human blood were obtained by the addio of 10, 100, or 1,000 parasites (epimastigote culture forms) of various *T. cruzi* clones. These samples were diluted with 1 ml of distilled water, boiled for 10 min, and centrifuged at 8,000 *g* for 10 min. The supernatants were boiled and centrifuged again at 8,000 *g* for 10 min. Blood samples from naturally infected patients were also processed in an identical manner. Ten microliter samples of the different extracts were used as templates in all PCR assays. After PCR, samples were electrophoresed on either 0.8% or 2% agarose gels, the gels were stained with ethidium bromide, and PCR subsamples (1/10 of the original sample) were analyzed.

PCR procedure

The oligonucleotides were obtained from P. Zalta (Centre de Recherche en Biologie et Genetique Cellulaire, Toulouse, France). Sequences were selected to anneal sites flanking the HVRm, as previously described[12] (5′-GATTGGGGTTGGAGTACTACTAT-3′ and 5′-TTGAACGGCCCTCCGAAAAC-3′). Two restriction endonuclease sites (*Sca* I and *Sau* 96 I) were artificially introduced at the 3′ end of each oligonucleotide to allow purification of the HVRm-amplified sequence from the oligonucleotide primers complementary to the conserved part of minicircle. Samples were amplified in 67 mM Tris HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mg/ml of bovine serum albumin, 75 pM of each deoxynucleotide triphosphate, and 75 pM of each oligonucleotide in a total reaction volume of 50 μl. A systematic aliquoting of this amplification buffer was done in 1.5-ml microtubes. Each aliquot was covered with 50 μl of paraffin to prevent evaporation, and was stored frozen at −20°C until use. *Thermus aquaticus* DNA polymerase (2.5 units) (Genofit, Geneva, Switzerland) was used for the amplification, which was performed with a Techne (Cambridge, UK) PH2 PCR device. The amplification procedure consisted of DNA denaturation (95°C for 5 min), oligoprimer annealing (48°C for 2 min), elongation (72°C for 2 min), 30 amplification cycles (95°C for 5 sec, 48°C for 30 sec, 70°C for 1 min), and cooling (20°C for 10 min).

Characterization of PCR-positive samples

The PCR-amplified 270 base-pair HVRm fragment was purified by electrophoresis on 0.8% preparative low melting point ultrapure agarose gels (Bethesda Research Laboratories, Uxbridge, UK). The fragment was eluted from the agarose using glass beads (Geneclean kit) according to the instructions of the manufacturer (Bio 101, La Jolla, CA USA), and then digested with the restriction endonucleases *Sau* 96 I and *Sca* I (Bethesda Research Laboratories) to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle. After digestion, the DNA was precipitated with ethanol, and resuspended in 100 μl of sterilized, distilled water.

TABLE 1

Direct characterization of Trypanosoma cruzi *clones in triatomine bug feces**

Origin of triatomine bugs	Number	Microscopic control	Positive by PCR	Natural clone no.
Endemic area	10	+	10	19, 20 (3 cases)
	12	−	2	
Xenodiagnosis	1	+	1	39 (1 case)
	35	−	1	43 (1 case)

* Natural clones are numbered according to ref. 1. PCR = polymerase chain reaction.

Non-radioactive labeling of purified HVRm (100 ng) was performed using digoxigenin (Boehringer, Mannheim, Germany) according to the instructions of the manufacturer. Amounts of DNA were quantitated using a Dipstick kit (Invitrogen, San Diego, CA). Samples (50 ng) of purified HVRm fragments were hybridized with DNA from various *T. cruzi* strains that were genetically characterized by isozyme analysis.[1] Briefly, total DNA from 1×10^4 parasites was amplified by PCR, electrophoresed on 0.8% agarose gels, alkali denaturated (two 15-min denaturations in 0.5 M NaOH, 1.5 M NaCl), and transferred onto charged nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK) by the pocket-blotting procedure.[16]

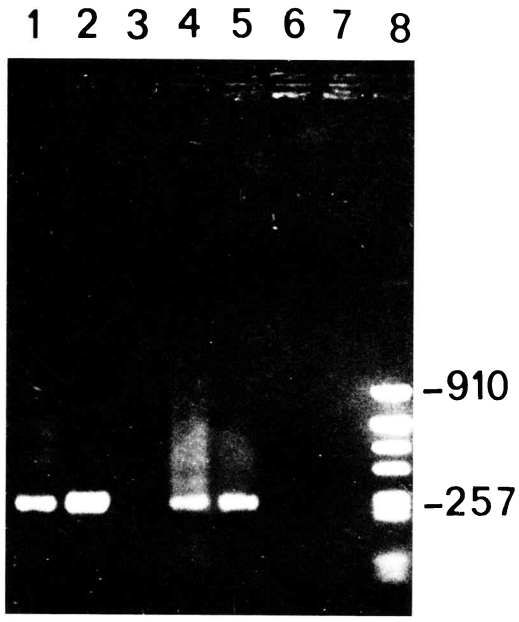

FIGURE 1. Ethidium bromide–stained 0.8% agarose gel containing polymerase chain reaction products of DNA isolated from a Bolivian triatomine bug fecal sample. Lanes 1, 2, 4, and 5, parasite-positive samples (positive by microscopic examination); lanes 3 and 6, parasite-negative samples (negative by microscopic examination); lane 7, control sample without template; lane 8, *Alu* I digest of pBR322. The amplified fragments have a length of approximately 270 base pairs. Values at right are size standards (basepairs).

RESULTS

Identification of natural clones from vectors

PCR sensitivity. Agarose gel electrophoretic analysis of PCR products from 58 triatomine fecal samples detected 14 positive specimens, as determined by visualization of a major intense band (270 bp) after staining the gels with ethidium bromide (Table 1 and Figure 1). All 11 fecal samples shown by microscopic examination to be infected with parasites were also positive by PCR analysis. Three triatomine specimens that were microscopically negative for parasites were PCR-positive. It is worth noting that one of these triatomes originated from a specimen that gave a negative result on a xenodiagnostic procedure. Of three other infected triatomes that had been collected in endemic areas, frozen, and shipped to our laboratory without cryoconservation, two were also PCR-positive (unpublished data).

Characterization of natural clones. Five PCR-positive samples from five triatomine fecal samples were tested. This procedure identified clones 39 (Figure 2A) and 43 (Figure 2B), and three isolates of either clones 19 or 20 (Figure 2C) (which are genetically related to each other). Isolates identified as clones 39 and 43 originated from xenodiagnosis of triatomine bugs. These clones had already been observed in Bolivian patients.[1, 17] The three other isolates stocks identified as clones 19 or 20 originated from triatomine bugs collected in an endemic area (the Yungas valley) where clone 20 had been already observed.[1, 18]

Detection of clones in experimentally-infected mouse blood

The results of PCR analysis of blood samples (100 μl) from a group of parasite-positive mice

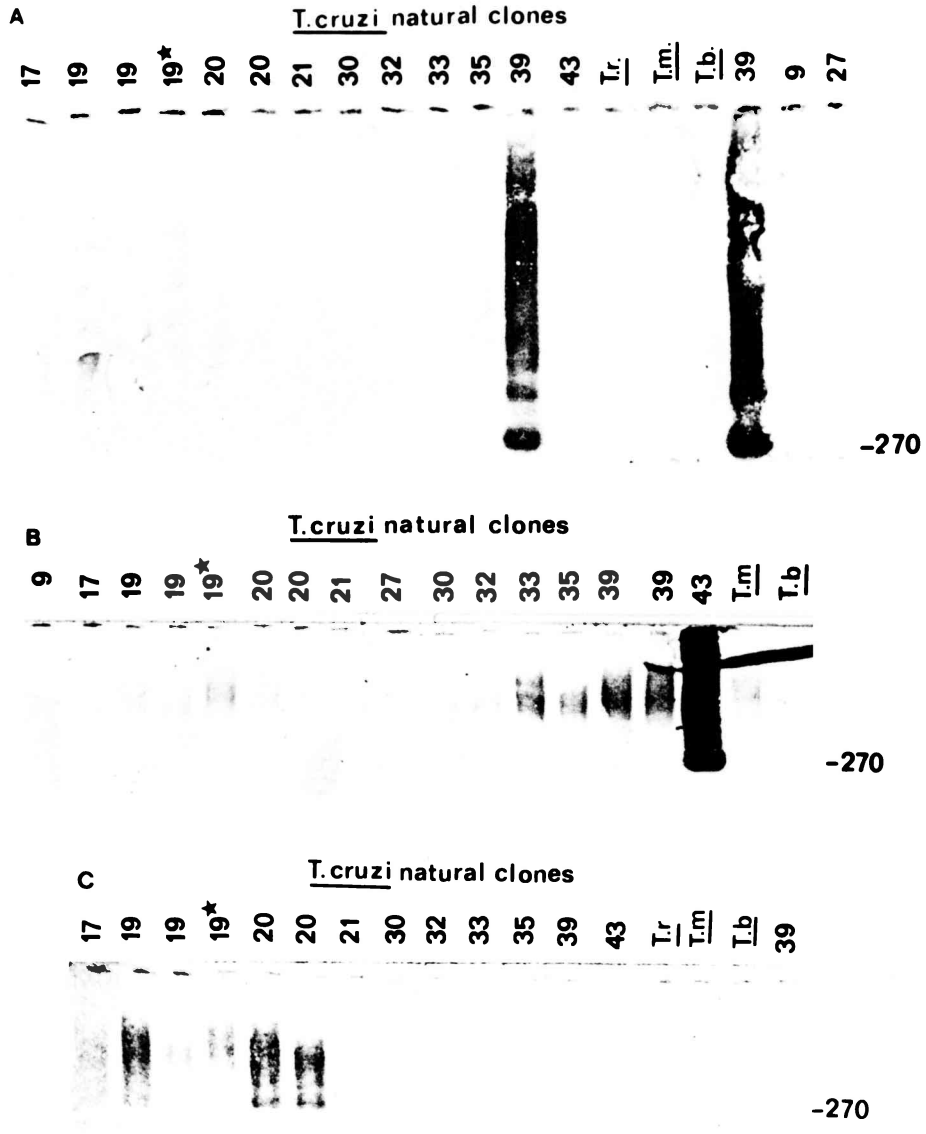

FIGURE 2. Hybridization patterns of three non-radioactively labeled highly variable region fragments of kinetoplast minicircle DNA from three different triatomine bug fecal samples obtained by polymerase (PCR) chain reaction amplification. These samples were hybridized with electrophoresed PCR products of total DNA of different *Trypanosoma cruzi* stocks that were transferred onto nylon membranes. The hybridization patterns were limited for the three different probes: 1) clone 39, **panel A,** lanes 12 and 17, TPK1 and bug 2146 stocks, respectively; 2) clone 43, **panel B,** lane 16, Tulahuen FKIIA c12 stock; 3) clones 19 or 20, **panel C,** lanes 2 and 3, clone 19 (Cutia, 13379 c17 stocks), lane 4, a clone closely related to clone 19 (19★) (LGN stock); lanes 5 and 6, clone 20 (Cuica, So34 c14 stocks). The other stocks of *T. cruzi* screened pertain to clones 9, 17, 21, 27, 30, 32, 33, and 35. *Trypanosoma rangeli* (T.r.), *T. marenkellei* (T.m.), and *T. brucei* (T.b.) control samples were also tested. Values at right are size standards (basepairs).

exhibiting either acute or chronic infections are shown in Table 2 and Figure 3A. All infected mice tested were PCR positive, except for animals in which parasitemia was less than 74 parasites/ml (no parasites were observed in 300 microscopic fields). This level of parasitemia appears to be the limit of detection under our experimental conditions. The hybridization of the

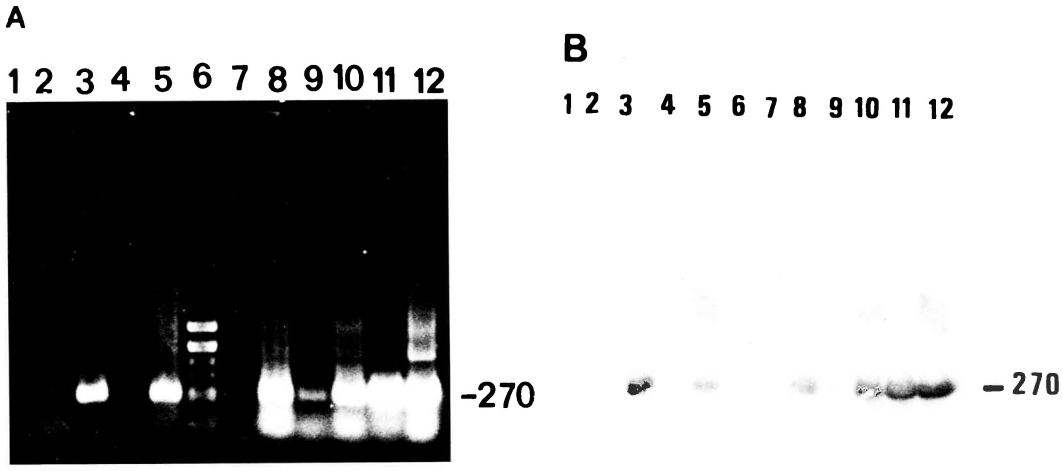

FIGURE 3. **A,** ethidium–bromide-stained 0.8% agarose gel containing polymerase chain reaction (PCR) products from DNA of mouse blood samples. **B,** hybridization pattern of these products with purified DNA from the highly variable region of the kinetoplast DNA minicircle of the Tulahuen c12 stock (clone 43) after transfer onto a nylon membrane. Lane 1, control sample (using distilled water as the template); lanes 2 and 7, uninfected mouse blood; lanes 3, 4, 5, 8, 9, and 12, blood from mice with a chronic infection at 190, 120, 120, 92, 140, 83 days, respectively, after infection; lanes 10 and 11, blood from mice in the acute phase of infection at 16 and 13 days, respectively, after infection; lane 6, *Alu* I digest of pBR322. Values at right are size standards (basepairs).

HVRm sequence with PCR products from mouse blood gave a pattern restricted to the Tulahuen c12 stock, which pertains to clone 43 (unpublished data). Moreover, HVRm purified sequences from the Tulahuen c12 stock and from triatomine bug fecal samples, both of which hybridize only with clone 43, showed strong hybridization patterns with PCR products from the different mouse blood samples (Figure 3B). Under the same conditions, no hybridization was observed with HVRm purified sequence from the Sc43 c11 stock pertaining to clone 39 (the most genetically related clone to clone 43 in our collection), for which the standard genetic distance to clone 43 is only 0.43.[1, 19]

Detection of clones in human blood

The modification used in PCR sample preparation of human blood (greater sample dilution; 100 μl of blood diluted in 1 ml of distilled water) was required to obtain a PCR-positive result (at least 10 parasites/100 μl of blood) (Figure 4A). The assay of artificially-infected blood samples were performed with different stocks (So34 c14 and Sc43 c11 pertaining to clones 20 and 39, respectively). Hybridization with the corresponding specific probes confirmed the sensitivity of this assay (Figure 4B). This sensitivity appears to be comparable with results obtained with mice blood. Under these conditions, samples of both chronically-infected (six cases) and non-infected (eight cases) Bolivian patients were tested, but none of these samples gave a positive result by PCR analysis.

DISCUSSION

These results clearly demonstrate the successful use of the HVRm sequences to identify different *T. cruzi* clones circulating in vectors and

TABLE 2

Direct detection of Trypanosoma cruzi *infection in acute and chronic cases of experimentally infected mice**

Days postinfection	Parasitemia (parasites/ml)	PCR result
13	2.74×10^6	+
16	3.40×10^6	+
83	7.40×10^2	+
92	2.95×10^2	+
120	$<0.74 \times 10^2$	−
120	1.48×10^2	+
140	$<1.40 \times 10^2$	+
190	5.18×10^2	+

* PCR = polymerase chain reaction.

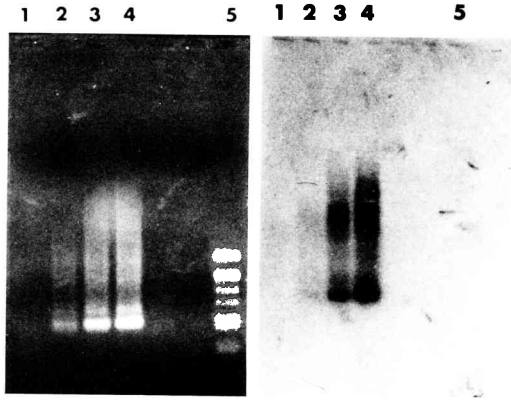

FIGURE 4. **A,** ethidium bromide–stained 0.8% agarose gel containing polymerase chain reaction (PCR) products from DNA of human blood samples (100 μl) artificially infected with the SC43 c111 stock (clone 39) of *Trypanosoma cruzi*. **B,** hybridization pattern of these products with purified DNA from the highly variable region of the kinetoplast DNA minicircle of the SC43 c11 stock after transfer to a nylon membrane. Lane 1, uninfected blood; lanes 2, 3, and 4, 100 μl of blood artificially infected with 10, 100, and 1,000 epimastigote culture forms, respectively; lane 5, *Alu* I digest of pBR322.

mammalian hosts. The preliminary results indicate that PCR sensitivity for vectors is quite satisfactory. Our results were consistent with microscopic evidence of parasite positivity, and in some instances, the PCR appears more sensitive than microscopic examination, which is expected. Therefore, the three samples that were parasite negative upon microscopic examination but parasite positive by PCR analysis were most likely infected triatomine bugs that presented with a very low level of parasitemia. The first consequence of these findings is the possibility of higher xenodiagnosis sensitivity. Our results indicate that xenodiagnosis, which is the most efficient method for parasitologic diagnosis of Chagas' disease, could be improved considerably by being used in conjunction with PCR analysis.

In addition, we were able to identify the parasitic clone circulating in five triatomine bugs by hybridization of amplified HVRm sequences with a set of *T. cruzi* clones that were fully characterized by isozyme analysis. These results are consistent with the available epidemiologic data concerning the various clones circulating in Bolivian patients and vectors. The clone specificity of the HVRm sequence has been clearly demonstrated for clones 39 and 20.[12] Some of our samples hybridized with both clones 19 and 20. Both of these clones are present in Bolivia.[1, 17, 18] One possible explanation for these non-specific hybridization patterns is the occurrence of mixed infections in vector studies already reported in Bolivia.[17, 20] There is also the possibility of cross-reacting hybridization of HVRm sequences from clone 19 with clone 20. Indeed, these clones are closely related, with a standard genetic distance of 0.02 (only one allelic difference recorded in 15 loci).[1]

The cross hybridization between the Tulahuen c12 stock and clone 43, which is the cloned stock used in the experimental infections as well as the stock circulating in a Bolivian patient, are consistent with isoenzyme multilocus characterization, and confirm the linkage between nuclear DNA and HVRm kinetoplast DNA evolution.[13]

The sensitivities for the detection of infection in mouse or human blood were comparable. Nevertheless, a weakly positive result must be interpreted cautiously, based on control samples (without DNA template) and hybridization results. This sensitivity level should be adequate enough in detecting acute human infections, where parasitemia levels are high, and this would make it possible to directly identify a specific clone in such cases. The assays results involving blood samples from chronic human infections were negative. In these cases, the small sample volume might be the main limiting factor for detection of positive PCR amplification. It should be emphasized that the classical xenodiagnosis test, which uses 30 L3 triatomine stages, requires approximately 2 ml of blood, while the present PCR experiments requires only 100 μl samples of blood.

The successful use of specific PCR probes under natural conditions (samples of blood and vector feces) will make it possible to establish a new standard for the comparison of the relevant medical properties (geographic distribution, clinical specificity, drug resistance, and virulence) of *T. cruzi* clones. This combination of a population genetics approach and a new technical tool provides an efficient means of investigating the long-standing, as yet unanswered, question regarding the medical consequences of *T. cruzi* genetic variability.

Financial support: This research was supported by EEC grant TSD 002/1608, INSERM grant 833013, and Region Languedoc-Rousillon grant 891891.

Authors' addresses: S. F. Breniere, M. F. Bosseno, and S. Revollo, Instituto Boliviano de Altura, ORSTOM-IBBA, Casilla 9214, La Paz, Bolivia. M. Tibayrenc, Laboratoire de Genetique des Parasites et des Vecteurs, ORSTOM, 2051 Av. du Val de Montferrand, BP 5045, 34032 Montpellier Cedex, France. M. T. Rivera and Y. Carlier, Laboratoire de Parasitologie, Universite Libre de Bruxelles (ULB), 115 bd. Waterloo, 1000 Bruxelles, Belgium.

Reprint requests: S. F. Breniere, ORSTOM-IBBA, Casilla 9214, La Paz, Bolivia.

REFERENCES

1. Tibayrenc M, Ayala FJ, 1988. Isoenzyme variability in Trypanosoma cruzi, the agent of Chagas' disease: genetical, taxonomical, and epidemiological significance. *Evolution 42:* 277–292.
2. Tibayrenc M, Solignac M, Cariou ML, Le Ray D, Desjeux P, 1984. Isoenzymic strains of Trypanosoma cruzi: recent or ancient, homogeneous or heterogeneous origin. *C R Acad Sci [III] 299:* 195–198.
3. Tibayrenc M, Ward P, Moya A, Ayala FJ, 1986. Natural populations of Trypanosoma cruzi, the agent of Chagas disease, have a complex multiclonal structure. *Proc Natl Acad Sci USA 83:* 115–119.
4. Miles MA, De Souza AA, Povoa M, Shaw JF, Lainson R, Toye PJ, 1978. Isozymic heterogeneity of Trypanosoma cruzi in the first autochthonous patients with Chagas' disease in Amazonian Brazil. *Nature 272:* 819–821.
5. Morel C, Chiari E, Plessmann Camargo E, Mattei DM, Romanha AJ, Simpson L, 1980. Strain and clones of Trypanosoma cruzi can be characterized by pattern of restriction endonuclease products of kinetoplast DNA minicircles. *Proc Natl Acad Sci USA 77:* 6810–6814.
6. Tibayrenc M, Kjellberg F, Ayala FJ, 1990. A clonal theory of parasitic protozoa: the population structures of Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas, and Trypanosoma and their medical and taxonomical consequences. *Proc Natl Acad Sci USA 87:* 2414–2418.
7. Tibayrenc M, Breniere SF, 1988. Typanosoma cruzi: major clones rather than principal zymodemes. *Mem Inst Oswaldo Cruz 83:* 249–255.
8. Tibayrenc M, Echalar L, Dujardin JP, Poch O, Desjeux P, 1984. The microdistribution of isoenzymic strains of Trypanosoma cruzi in southern Bolivia: new isoenzyme profiles and further arguments against Mendelian sexuality. *Trans R Soc Trop Med Hyg 78:* 519–525.
9. Carrasco R, Breniere SF, Poch O, Miguez H, Selaes H, Antezana G, Desjeux P, Carlier Y, 1985. Chagas serology and its problems. *Ann Soc Belg Med Trop 65 (suppl 1):* 79–84.
10. Carneiro N, Chiari E, Goncalves AN, da Silva Pereira AA, Morel CM, Romanha AJ, 1990. Changes in the isoenzyme and kinetoplast DNA patterns of Trypanosoma cruzi strains induced by maintenance in mice. *Acta Trop 47:* 35–45.
11. Sanchez G, Wallace A, Olivares N, Aguilera X, Apt W, Solari A, 1990. Biological characterization of Trypanosoma cruzi zymodemes in vitro: differentiation of epimastigotes and infectivity of culture metacyclic trypomastigotes to mice. *Exp Parasitol 71:* 125–133.
12. Veas F, Cuny G, Breniere SF, Tibayrenc N, 1991. Subspecific kDNA probes for major clones of Trypanosoma cruzi. *Acta Trop 48:* 79–82.
13. Tibayrenc N, Ayala FJ, 1987. Forte correlation entre classification isoenzymatique et variabilite de l'ADN kinetoplastique chez Trypanosoma cruzi. *C R Acad Sci [III] 304:* 89–92.
14. Breniere SF, Carrasco R, Miguez H, Lemesre JL, Carlier Y, 1985. Comparisons of immunological tests for serodiagnosis of Chagas' disease in Bolivian patients. *Trop Geogr Med 37:* 231–238.
15. Carlier Y, Rivera MT, Truyens C, Puissant F, Milaire J, 1987. Interactions between chronic murine Trypanosoma cruzi infection and pregnancy: fetal growth retardation. *Am J Trop Med Hyg 37:* 534–540.
16. Cuny G, Veas F, Roizes G, 1991. Pocket-blotting: a method for tranferring nucleic acid onto nylon membrane. *Anal Biochem 193:* 45–48.
17. Breniere SF, Carrasco R, Revollo S, Aparicio O, Desjeux P, Tibayrenc M, 1989. Chagas'disease in Bolivia: clinical and epidemiological features and zymodeme variability of Trypanosoma cruzi strains isolated from patients. *Am J Trop Med Hyg 5:* 521–529.
18. Breniere SF, Braquemond P, Solari A, Agnese JF, Tibayrenc M, 1991. An isoenzyme study of naturally occurring clones of Trypanosoma cruzi isolated from both sides of the west Andes highland. *Trans R Soc Trop Med Hyg 85:* 62–66.
19. Nei M, 1972. Genetic distances between populations. *Am Naturalist 106:* 282–292.
20. Tibayrenc M, Cariou ML, Solignac M, Dedet JP, Poch O, Desjeux P, 1985. New electrophoretic evidence of genetic variation and diploidy in Trypanosoma cruzi, the causative agent of Chagas' disease. *Genetica 67:* 223–230.