

## *Trypanosoma cruzi*: Parasite Detection and Strain Discrimination in Chronic Chagasic Patients from Northeastern Brazil Using PCR Amplification of Kinetoplast DNA and Nonradioactive Hybridization

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Laboratory of Molecular Biology and Endemic Diseases, Department of Biochemistry and Molecular Biology, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, 21045-900 Rio de Janeiro, Brazil; \*Laboratoire de Parasitologie, Faculté de Médecine, 163 rue A. Broussonet, 34000 Montpellier, France; and †Department of Tropical Medicine, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, 21045-900 Rio de Janeiro, Brazil

BRITTO, C., CARDOSO, M. A., RAVEL, C., SANTORO, A., BORGES PEREIRA, J., COURA, J. R., MOREL, C. M., AND WINCKER, P. *Trypanosoma cruzi*: Parasite detection and strain discrimination in chronic chagasic patients from northeastern Brazil using PCR amplification of kinetoplast DNA and nonradioactive hybridization. *Experimental Parasitology* 81, 462-471. Blood samples from 172 individuals from northeastern Brazil were subjected to PCR amplification of *Trypanosoma cruzi*-specific kDNA sequences. This method enabled us to detect parasite DNA in 21 of 47 patients that were serologically positive. In addition, 1 patient that gave doubtful results with chagasic serology was confirmed as positive by PCR. We applied the same PCR detection method to the feces of wild triatomines captured in the same region, obtaining three positive results that were confirmed by microscopic examination. The 25 amplified products obtained in this study were then reamplified with primers that gave a final amplicon containing sequences from the most variable region of kDNA minicircles. These were used as probes in hybridization experiments aimed at defining the degree of relatedness between the strains infecting humans and insects based on kDNA homologies. We found that the amplification products from the three triatomines were related and showed no cross-hybridization with those obtained from human infections. Eight amplified products from human infections showed no cross-hybridization and did not hybridize with products from other patients. This indicates that the strains of *T. cruzi* circulating in the region present a high level of genetic heterogeneity. Finally, a number of amplified products hybridized with amplicons that did not hybridize with each other, indicating that infections with a parasite population presenting a mixed kDNA content (either due to different strains of *T. cruzi* or to a hybrid parasite) are a more frequent event than previously thought. © 1995 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Trypanosoma cruzi*; PCR diagnosis; schizodeme; kDNA hybridizations; Chagas' disease; molecular epidemiology; polymerase chain reaction (PCR); kinetoplast deoxyribonucleic acids (kDNA); base pairs (bp); sodium dodecyl sulfate (SDS); ethylene diamine tetraacetic acid (EDTA).

### INTRODUCTION

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is endemic in many regions of Latin America. Due to the low parasitemia in patients with chronic infection, its diagnosis relies mainly on the detection of antibodies against *T. cruzi* antigens (Camargo 1992). The major limitation of these serological techniques is their specificity, principally be-

cause *T. cruzi* antigenic fractions share cross-reactive epitopes with those of other trypanosomatids present in the same endemic areas, in particular *Leishmania* sp. (Araujo 1986). No case of self-cure has been demonstrated for Chagas' disease, and therefore every serology-positive individual is considered to have a current infection. It has been suggested that serological methods should be validated by parasitological diagnosis. However, the low sensitivity of the currently used parasite detection techniques, such as hemoculture and xenodiag-

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nosis, make them of limited use for confirming Chagas' disease (Chiari *et al.* 1989; Coura *et al.* 1991).

To overcome this problem, substantial efforts have been invested in the development of methods for detecting *T. cruzi*-specific DNA in blood samples using the polymerase chain reaction (PCR). One of the systems developed for this purpose is based on specific amplification of kinetoplast minicircle DNA, a constituent of the trypanosome mitochondrion (Sturm *et al.* 1989). The target sequences for the PCR reaction are present in very high copy numbers per cell, and reconstitution experiments have shown that under experimental conditions this technique is capable of detecting the equivalent of one parasite in 20 ml of blood (Avila *et al.* 1990).

The next step in the validation of PCR-based diagnosis of Chagas' disease is the evaluation of its sensitivity and specificity in clinical samples, which requires that large numbers of patients be tested. Two large-scale studies have been conducted in one city of southeastern Brazil, leading to the conclusion that the sensitivity of PCR is 96.5–100%, in comparison with serological diagnosis (Avila *et al.* 1993; Wincker *et al.* 1994). However, Chagas' disease displays a wide variety of clinical manifestations, including in terms of parasitemia levels, depending on the endemic region under study (Coura *et al.* 1984). Therefore, the use of PCR-based diagnosis should be evaluated in different geographical situations. One of the aims of the present study was to compare the efficacy of PCR diagnosis with classical serological techniques in a region of northeastern Brazil with strikingly different epidemiological characteristics compared to the region where the first studies were carried out.

Another potential application of PCR detection of *T. cruzi* DNA in blood samples is the use of the amplified product as a molecular marker in strain characterization. Our protocol for kDNA amplification yields a product derived from the minicircle variable region, a highly polymorphic sequence (Degraeve *et al.* 1988). These sequences have proven useful in strain

typing of *T. cruzi* either by DNA restriction digests patterns (schizodeme analysis, Morel *et al.* 1980) or by hybridization (Avila *et al.* 1990; Breniere *et al.* 1992). A commonly occurring problem in the use of molecular markers as tools for characterizing *T. cruzi* strains in infected patients is the selection that inevitably occurs when isolating and growing cells from an initial isolate. Cell culture is necessary to produce sufficient amounts of *T. cruzi* for characterization either by isoenzyme typing (zymodeme analysis) or by schizodeme analysis. In contrast, since the PCR products from blood sample amplifications represent the whole population of parasite cells, we reasoned that they may be useful for typing purposes. The present work investigated this possibility by performing cross-hybridizations between PCR products amplified from chagasic patients and naturally infected triatomines from a defined endemic region.

#### MATERIALS AND METHODS

##### *Endemic region under study and inhabitants examined.*

All the individuals participating in the present study lived in the Sertão da Paraíba region in the northeast of Brazil, where Chagas' disease is endemic (Borges Pereira and Coura 1987). This region has been studied by the Department of Tropical Medicine of the Oswaldo Cruz Foundation for the last 15 years. Domestic transmission was stopped in this region about 10 years ago by systematic insecticide spraying; therefore, all the chagasic patients we studied were in the chronic phase of the disease. They lived in four different cities and donated blood in February, 1994, during an investigation carried out by the Oswaldo Cruz Foundation in the region.

*Serological diagnosis.* Serum was prepared from the blood of each patient, and three different tests were performed: indirect immunofluorescence using epimastigotes of the Y strain of *T. cruzi* as antigens, enzyme-linked immunosorbent assay (ELISA) using a Y strain cytosolic fraction, and an ELISA test using two recombinant proteins from *T. cruzi* (Krieger *et al.* 1992). A patient was considered serology-positive when all three tests were positive, doubtful when only one or two tests were positive, and negative when all three techniques gave a negative result.

*DNA preparation for PCR.* Five milliliters of blood was collected from each patient and immediately mixed with an equal volume of a solution of 6 M guanidine hydrochloride and 0.2 M EDTA (Avila *et al.* 1991). The samples were stored at room temperature for 5–15 days and subsequently at 4°C. When they reached the Rio de Janeiro laboratory, they were immersed in a boiling water bath for 15 min to

shear the DNA molecules (Britto *et al.* 1993). After cooling, two 100- $\mu$ l aliquots were taken from each patient's blood lysate, and successive phenol-chloroform and chloroform extractions were performed on this material. After a final ethanol precipitation, the pellet was resuspended in 50  $\mu$ l of distilled water.

**PCR conditions.** The "hot-start" protocol was used in all the amplification reactions, with the use of an Ampliwax Gem (Perkin-Elmer) to separate the two phases (Chou *et al.* 1992). The lower phase consisted of 5  $\mu$ l of 10 $\times$  *Taq* DNA polymerase buffer, 7.2  $\mu$ l of a 10 mM dNTPs mixture, 13.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 200 ng of the *T. cruzi*-specific primers (No. 121 5'AAATAATGTACGGG(T/G)GAGATGCATGA3' and No. 122 5'GGTTCGATTGGGGTTGGTGTAATATA3'), and 27.3  $\mu$ l of water; the upper phase consisted of 7.5  $\mu$ l of the DNA sample, 2.5  $\mu$ l of 10 $\times$  *Taq* DNA polymerase buffer, 2.5 units of *Taq* DNA polymerase, and water to 25  $\mu$ l. The cycling parameters were: 2 cycles at 98°C for 1 min and 64°C for 1 min, 33 cycles at 94°C for 1 min and 64°C for 1 min, and a final incubation at 72°C for 10 min. The samples that remained negative for minicircle-specific amplification were further tested under the same conditions with two oligonucleotides from the human  $\beta$ -globin gene region (No. PC03 5'ACACAACTGTGTCTACTAGC3' and No. PC04 5'CAACTTCATCCACGTTCCACC3') to discard the possibility that a negative result could have occurred due to inhibition of the PCR reaction (Wincker *et al.* 1994). The same protocol was used for the reamplifications that provided the products used in the hybridization reactions, except that primer No. 121 was replaced by No. 256 5'AAATTTGA(A/C)(G/C)(G/C)CCCTCCCCA3'. A series of precautions were taken to avoid contamination of the preparations and reaction mixtures, including the use of a laminar flow hood during the preparation step, the physical separation of the areas used for different stages of analysis, the use of different sets of micropipettes for each function, and the use of tips with filters. The possibility of contamination of the PCR reagents and of the solutions used for preparing DNA was checked with appropriate controls (Wincker *et al.* 1994), and each sample was tested in duplicate.

**DNA hybridizations.** All hybridizations were carried out using a reamplified fragment of approximately 290 bp, obtained from a PCR reaction using as substrate the original product (amplified with the primers Nos. 122 and 121), and as primers the oligonucleotides Nos. 122 and 256. The reamplified products were electrophoresed on a 2% agarose gel and then transferred to a Genebind 45 nylon membrane (Pharmacia). The membranes were prehybridized in 5 $\times$  SSPE (1 $\times$  SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA, pH 7.4), 5 $\times$  Denhardt's solution, 0.5% SDS, and 20  $\mu$ g/ml denatured salmon sperm DNA at 65°C and hybridized in the same solution with 5 ng/ml of the biotinylated probe (consisting of the same reamplified 290-bp products without purification) labeled using the BioPrime labeling kit (Life Technologies). Washing was carried out in 0.1 $\times$  SSPE at 65°C. The development of the results was done using a streptavidin-alkaline phosphatase conjugate

and a chemiluminescent substrate (Photogene Detection System, Life Technologies) following the manufacturer's instructions. One hour after the addition of the chemiluminescent reagent, the membranes were exposed to X-ray films for 2 min.

## RESULTS

**Comparison of PCR diagnosis with serological methods.** Of the 172 individuals living in the endemic area who were submitted to serological diagnosis for Chagas' disease, 47 gave a positive result with all three used techniques, while 40 were positive with only one or two of them. When submitted to PCR diagnosis, 21 of the 47 serology-positive patients were detected, while only 1 of the 40 doubtful individuals gave a positive amplification result (Table I). Finally, none of the 85 blood samples from serology-negative individuals gave any amplification by PCR. We further tested our PCR test with purified DNA from local endemic *Leishmania* species. No product was amplified using as substrates DNA from isolates of *L. chagasi*, *L. guyanensis*, *L. braziliensis*, or *L. amazonensis* (not shown).

**Detection of *T. cruzi* in wild triatomines by PCR.** One hundred and twenty wild triatomines were captured in the proximity of an area of habitation situated at the edge of the main road of the Sertão da Paraíba region. They belong to the species *Triatoma braziliensis*, the main vector of Chagas' disease in the region. They were stored alive in 12 boxes of 10 triatomines each and sent to the Rio de Janeiro laboratory for further examination. The feces from each box were collected in 12 pools and submitted to DNA extraction and PCR amplification with the *T. cruzi*-specific oligonucleotides Nos. 121 and 122. Three of these pools gave a positive am-

TABLE I  
Comparison of the PCR Diagnosis Results with Serology

Patient status	Patient number	PCR	
		Positive	Negative
Serology-positive	47	21	26
Serology-doubtful	40	1	39
Serology-negative	85	0	85

plication result (Fig. 1). Subsequent microscopic examination of the feces from each triatomine of the positive pools revealed that a single insect was infected in each case. No parasite was detected by microscopy in the PCR-negative pools.

*Use of the amplified products as hybridization probes for characterization of the infecting strains.* In order to analyze the relationships between the *T. cruzi* strains circulating in the Sertão da Paraíba region, we decided to test the utility of the amplified products obtained in the diagnosis experiments in hybridization studies. We observed that when using the 330-bp products as probes, we consistently obtained a low level of cross-hybridization in every experiment, even when using PCR amplicons of patients from other geographic regions (not shown). This is probably due to the presence of sequences in the amplicon from the conserved region of the minicircle, which were of sufficient size to give stable duplexes. To overcome this problem, a reamplification step was introduced using primers Nos. 122 and 256, which produced an amplified product of 290 bp with a minimal content of minicircle conserved regions. In addition, all the wash conditions were very stringent ( $0.1 \times$  SSPE,  $65^\circ\text{C}$ ), so that only

the products derived from very close strains can cross-hybridize. We performed a test experiment with two probes of 290 bp from two isolates of the Sertão da Paraíba. We did not observe any cross-hybridization of these probes with three laboratory strains (Y, CL, and Dm 28), nor with 6 patients from distant endemic areas (not shown). These reamplified products were used in all the experiments described below. We first tested the distribution of the strains of *T. cruzi* that infected the three wild triatomines previously described. Using each one successively as a probe against all the 25 amplified products obtained in the region (3 from the insects and 22 from the chagasic patients) we observed that the three triatomine-derived probes cross-reacted strongly, indicating that the insects carried a common infecting strain (or strains). In contrast, none of the amplicons derived from the human infections reacted with these probes (Fig. 2). Next, each one of the 22 amplicons derived from chagasic patients was labeled with biotin and used as a hybridization probe against the other amplified products. Four different types of results were observed. In the first case, a probe hybridized solely with the amplicon from which it was derived (Fig. 3). In other cases, an amplicon recognized only one additional product, with the same result being obtained in a reverse hybridization experiment (not shown). In both cases, we concluded that the *T. cruzi* strains from which the amplicons originated were not present in a large fraction of our sample, nor did they participate in obvious mixed infections. The number of non-cross-hybridizing strains corresponding to such cases was 8 for a total of 22 individuals examined (see products P11, P31, P46, P84/P117, P116, P120, P435/439, and P446 in Table II). A third situation occurred when an amplicon used as a probe hybridized with amplified products from different patients, while these latter products failed to cross-hybridize with each other when used as probes. An example is shown in Fig. 4. It can be seen that the amplicon from patient 92 is recognized by those from patients 68, 75, and 161, but that these three latter products share no detectable

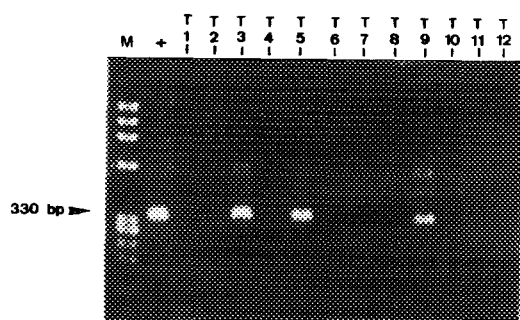


FIG. 1. Amplification results from 12 pools of feces of 10 wild triatomines each. M, molecular weight marker,  $\phi$ X 174 DNA cut by *Hae*III. (+) Positive control (DNA prepared from the blood of a chagasic individual with positive PCR). T1 to T12, amplified products from the 12 triatomine feces pools. All samples were amplified for 35 cycles with the minicircle specific primers Nos. 121 and 122 as described under Materials and Methods. The products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

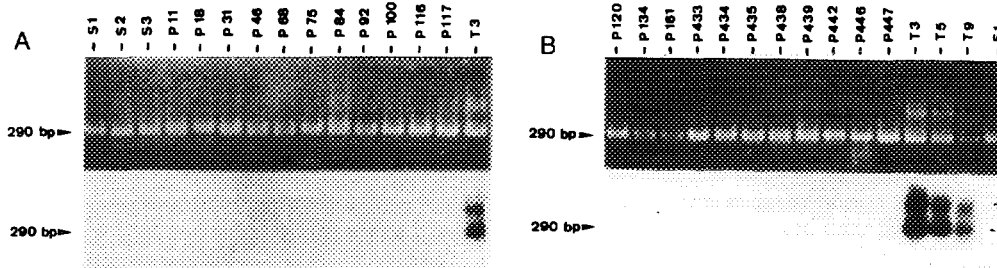


FIG. 2. Hybridization of the triatomine-derived T3 product against the amplified products derived from chagasic patients and from other triatomines. S1 to S3, amplified products from three chagasic individuals living in an endemic region distant from the Sertão da Paraíba. All samples noted Px were amplified from chagasic patients of the Sertão da Paraíba region. T3, T5, and T9 are the amplified products from the three positive triatomine feces pools. All the products originated from a reamplification of the 330-bp original amplicons with the primers Nos. 122 and 256 (A and B). Two different agarose gels (top part) that had been transferred and hybridized with the T3 amplified product as probe (bottom part).

homology. These results indicate the presence in patient 92 of a mixed infection, or of a hybrid trypanosome strain. Finally, in some other cases, an amplicon hybridized with a series of products from different patients, which themselves hybridized with a number of other, different, products, but with a nonoverlapping pattern (Fig. 5 and Table II). In some of these cases, a mixed component in the infection is certain (see, for example, patient 134 in Table II, which cross-reacts with the probes derived from patients 100 and 18, while these two last

probes do not cross-hybridize). However, in all these cases, the complexity of the patterns observed could not be completely resolved with the number of probes at our disposal.

#### DISCUSSION

In the present study, the polymerase chain reaction allowed us to detect *T. cruzi* kDNA in the blood of 45% of the patients from the Sertão da Paraíba region who were serodiagnosed as chagasic. Using the same method, we detected 96.5% of the serology-positive patients in Vir-

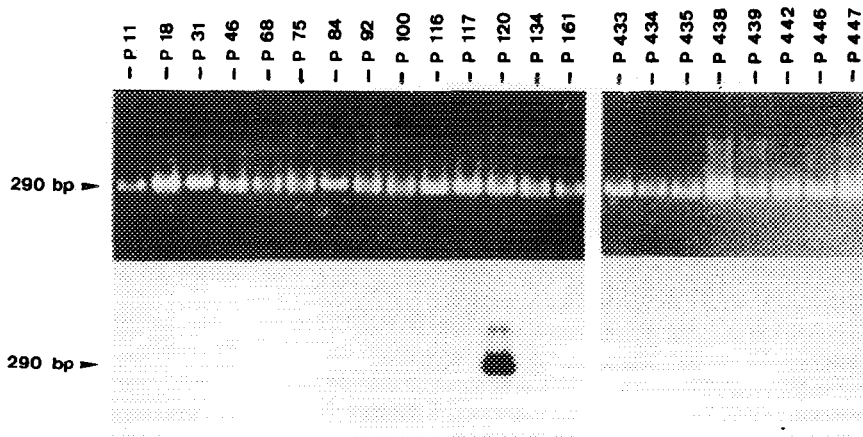


FIG. 3. Hybridization of the amplified product from the chagasic patient 120 with the amplified products from all the PCR-positive chagasic patients from the Sertão da Paraíba region. Px represents the product obtained with the primers Nos. 122 and 256 by reamplification of the original 330-bp product from patient x. The upper part of the figure shows the ethidium bromide-stained 2% agarose gel on which the amplicons were electrophoresed. The bottom part shows the result of the hybridization, aligned with the top photograph.

TABLE II  
Cross-hybridization Results between the Products Amplified from the 22 Patients' Blood Samples

Probes	Blotted DNA																						
	P11	P18	P31	P46	P68	P75	P84	P92	P100	P116	P117	P120	P134	P161	P433	P434	P435	P438	P439	P442	P446	P447	
P11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P18	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P31	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P46	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P68	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P75	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P84	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P92	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P100	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P116	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P117	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
P120	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
P134	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
P161	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P433	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
P434	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
P435	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
P438	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
P439	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
P442	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
P446	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
P447	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Note: The notation Px indicates the product amplified from patient No. x.

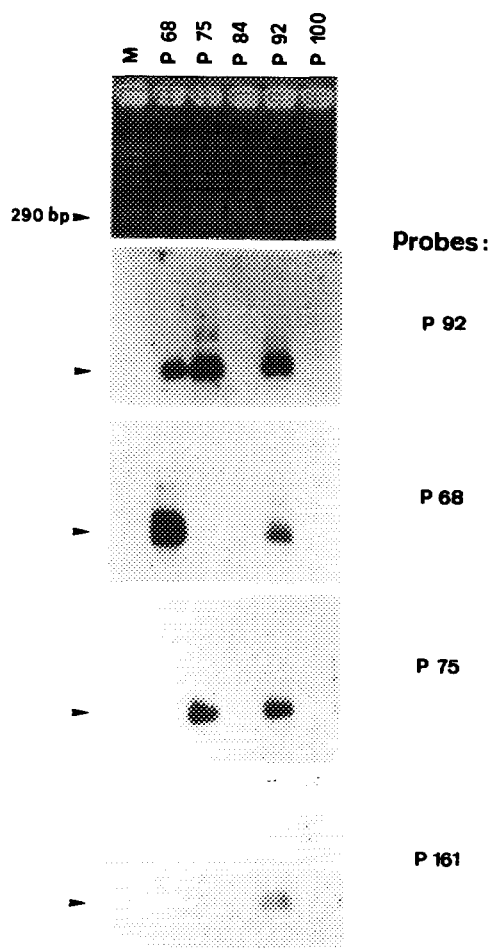


FIG. 4. Example of a chagasic patient infected with a *Trypanosoma cruzi* population presenting a mixed kDNA constituent. All the amplified products were obtained through PCR amplification using the oligonucleotides Nos. 122 and 256 and the original 330-bp products as substrates. M, molecular weight marker ( $\phi$ X 174 DNA digested with *Hae*III). The top part of the figure shows the agarose gel on which were electrophoresed the amplified products from five patients that were PCR-positive and lived in the same city. The hybridization results obtained with four different probes that reacted with the product amplified from patient 92 are shown below. The arrowheads point to the 290-bp reamplified products.

gem da Lapa, an endemic city southeastern Brazil (Wincker *et al.* 1994). These results clearly indicate that the efficiency of the PCR diagnosis method varies in relation to the endemic region under study. It should be noted that the results

we obtained paralleled the differences in sensitivity observed in previous studies for xenodiagnosis when compared to serology, namely, 43% for Virgem da Lapa (Borges Pereira *et al.* 1984) and only 13% for the Sertão da Paraíba (Borges Pereira and Coura 1987). However, the sensitivity of the PCR method was substantially higher than that of the xenodiagnosis in both situations, suggesting that it is the best available technique for *T. cruzi* detection in blood samples from chronic patients from different endemic areas.

The PCR results obtained with serologically doubtful patients were in strong contrast with those from the serology-positive individuals. While 45% of the latter were PCR-positive, only 1 in 40 of the serologically undefined patients gave a positive amplification result. This suggests that most of the doubtful results arose from problems in the specificity of serological methods in this region, perhaps due to cross-reaction with antigens from other parasites. Whether other infections are indeed responsible for the positive results obtained using some serological tests in these cases will require further investigation. Finally, we obtained a 100% specificity of the PCR technique when compared to serology, confirming the result obtained in the Virgem da Lapa study (Wincker *et al.* 1994).

The use of the amplified products derived from kDNA variable region sequences as hybridization probes allowed us to compare the strains infecting chagasic patients. Due to the high rate of mutation of these sequences, one can assume that two cross-hybridizing products were amplified from closely related *T. cruzi* strains (Simpson 1987). In a previous, more limited survey, Veas *et al.* (1991) demonstrated that two probes derived from minicircle variable regions from two cloned strains of *T. cruzi* hybridized only with strains belonging to the same zymodemes. This indicates that the resolving power of this method is high and that it may probably find more use in analyzing the diversity of *T. cruzi* in a limited endemic area than as a general method of comparison between distantly related strains. The relationships

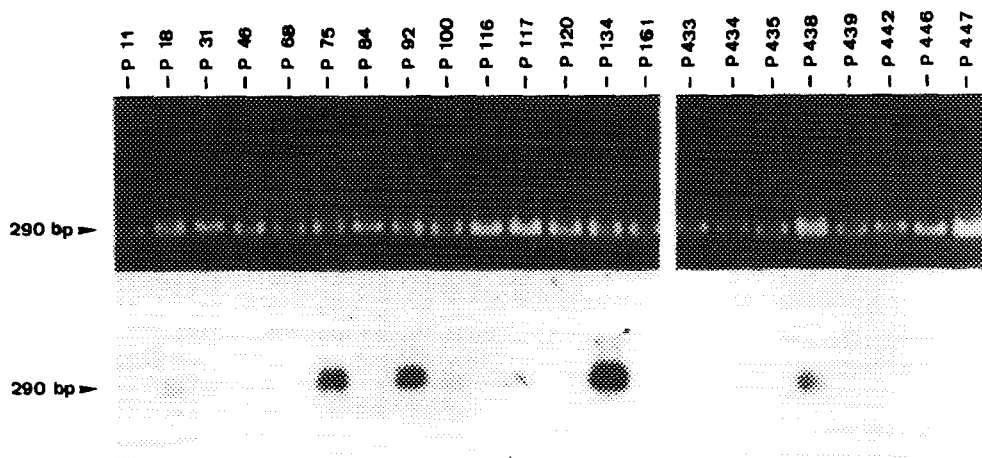


FIG. 5. Hybridization of the amplified products from chagasic patient 134 against the amplified products from all PCR-positive patients from the Sertão da Paraíba region. Px represents the product obtained with the primers Nos. 122 and 256 using the original 330-bp product from patient *x* as template. The upper part of the figure shows the ethidium bromide-stained 2% agarose gel on which the amplicons were electrophoresed. The bottom part shows the result of the hybridization, aligned with the top photograph.

between zymodemes and strains with cross-hybridizing minicircle variable regions remain hypothetical and will need to be investigated in a large-scale comparative study.

We found that the use of probes with a minimal content of minicircle conserved sequences allowed clear differentiation between hybridizing and nonhybridizing products. Previous work using total kDNA or cloned minicircles as probes, although useful, gave results which were difficult to interpret, principally in the cases of a low-intensity signal (Macina *et al.* 1987). Other difficulties occurred when using total kDNA, or even PCR-amplified minicircle variable regions, in fingerprinting analysis after restriction enzyme digestion. In these cases, the pattern is always complex and the comparisons are limited to the most obviously identical fingerprints (Gonçalves *et al.* 1984; Avila *et al.* 1990). As the method described in the present paper bypasses both types of problems, it may become the method of choice for comparing kDNA contents of *T. cruzi* isolates. A next step to determine the degree of variation between strains would be the primary sequence analysis of these PCR products.

The cross-hybridization results obtained in

this study revealed that for 25 isolates from the Sertão da Paraíba region, we can differentiate at least 8 amplified products that did not cross-hybridize. In addition, we observed a series of nonoverlapping patterns of hybridization among strains that gave a more complex result, suggesting the existence of additional strains with a different kDNA content. This indicates considerable genetic heterogeneity of *T. cruzi* in this particular region. Many lines of evidence point to the old origin of *T. cruzi* in the Sertão da Paraíba, including the fact that its insect vector there, *T. braziliensis*, is natural to the region, by contrast with many endemic zones where the recently introduced *Triatoma infestans* is the main domiciliary vector species (Dias 1992). A very different picture is indeed obtained where *T. infestans* predominates, and where a more limited number of *T. cruzi* strains seems to be present (Tibayrenc and Ayala 1988). It would be interesting to investigate if recent vector migrations have been accompanied by *T. cruzi* strain selection.

An important finding of the present work is the presence of *T. cruzi* kDNA in some patients that hybridized with amplified products from different isolates that do not share homology. It



is not possible, with the data available, to decide if this is due to the presence of a mixed infection or to the existence of a *T. cruzi* strain with a hybrid kDNA content. Data on population structure of *T. cruzi* indicate that recombination may be extremely rare in this species (Tibayrenc *et al.* 1986) and that mixed populations may not be uncommon in some regions (Morel *et al.* 1986). As we detected many cases of nonoverlapping patterns of cross-hybridization in this study, the most probable explanation may be that mixed infections are frequent. These data contrast with those of a previous report that used total kDNA and cloned minicircles to detect homologies between *T. cruzi* strains in Argentina and Chile (Macina *et al.* 1987). In this study, a single case of mixed infection was found in 52 isolates from wild triatomines and human isolates. Three explanations can be found for this discrepancy: first, only seven probes were used. Although some of these probes recognized a high number of parasites, this is an insufficient number to analyze all the homologies between all isolates. Second, the substrates for hybridization were purified kDNA networks, prepared after a step of cell culture. This could have led to selection of the fastest-growing strains. Finally, these discrepancies may occur due to natural differences in the pattern of infection in each region studied.

Taken together, the hybridization results of the present study suggest that PCR detection of kDNA in biological samples may be the most powerful available technique to study the complexity of *T. cruzi* infections in natural situations and the molecular epidemiology of Chagas' disease.

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