

Detection of *Trypanosoma cruzi* in Blood Specimens of Chronic Chagasic Patients by Polymerase Chain Reaction Amplification of Kinetoplast Minicircle DNA: Comparison with Serology and Xenodiagnosis

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A panel of 114 blood samples from chronic chagasic patients and nonchagasic patients was screened for *Trypanosoma cruzi* by xenodiagnostic, serologic, and polymerase chain reaction (PCR) amplification tests. Blood samples were preserved in a guanidine-EDTA buffer, and total blood DNA was isolated after chemical nuclease cleavage with 1,10-phenanthroline-copper ion and used as a template for PCR amplification of the conserved and variable regions of *T. cruzi* minicircle molecules. The PCR products were screened by Southern blot hybridization with a digoxigenin-labeled oligonucleotide probe specific for the conserved region of the minicircle. The method showed a sensitivity of 100% compared with the serologic test. In addition, all of the serology-positive, xenodiagnosis-negative samples were positive by PCR. This demonstrates that PCR amplification of *T. cruzi* kinetoplast minicircle DNA could replace xenodiagnosis for evaluation of parasitemia in chronic chagasic patients and could serve as a complement for serologic testing in the screening of blood bank donors.

Chagas' disease is a major cause of morbidity and mortality in Latin America. The etiological agent, *Trypanosoma cruzi*, is transmitted by triatomid bugs. However, human transmission can occur by alternative routes, such as blood transfusion and congenital infection. During the initial, acute phase, direct detection of the parasite in blood smears is possible because of the high levels of parasitemia. In contrast, detection of *T. cruzi* during the lifelong chronic phase requires biological amplification methods, such as hemoculture and xenodiagnosis, because of the low levels of parasitemia. Xenodiagnosis represents the most sensitive and specific assay for direct detection of *T. cruzi* in blood of chronic chagasic patients. However, xenodiagnosis yields positive results in only 17 to 70% of serologically positive patients (18). Serologic tests for diagnosis of chronic Chagas' disease have high sensitivity but lack specificity because of antigenic cross-reactivity with other parasites, such as *Leishmania* sp. and *T. rangeli* (12, 17). In addition, serologic assays only detect antibodies against the parasite and not the presence of the parasite itself. This is an important consideration when deciding what tests to use to monitor cure rates in patients undergoing drug treatment for Chagas' disease.

We have previously developed a method for the lysis and storage of whole blood specimens at room temperature and a procedure for the isolation of kinetoplast DNA (kDNA) from these blood lysates with subsequent polymerase chain reaction (PCR) amplification of kDNA minicircle sequences (5). Briefly, the procedure is as follows. Whole blood is stored at room temperature as a lysate in 3 M guanidine HCl-0.1 M EDTA. DNA stored in these guanidine-EDTA-blood lysates remains undegraded for 1 month at 37°C. *T. cruzi* minicircle DNA in the blood lysate is randomly cleaved

by using the chemical nuclease 1,10-phenanthroline-copper ion. This procedure liberates minicircle molecules from catenation in networks, thereby distributing them throughout the lysate and allowing a small aliquot of the original lysate to be analyzed for minicircle sequences. We showed that the presence of excess human DNA has no effect on the amplification of parasite minicircle DNA (5, 19) and that kDNA minicircle sequences represent appropriate species- and strain-specific markers for detection and classification of *T. cruzi* (4, 19). The 1.4-kb *T. cruzi* minicircle molecules are organized into four segments, each consisting of a short DNA region with a high level of sequence conservation among all *T. cruzi* minicircles and a longer DNA region exhibiting high sequence variability (8). Primers within the conserved regions were used to PCR amplify the variable regions bracketed between them, generating a 330-bp PCR product (4, 19). The extensive DNA sequence heterogeneity observed in *T. cruzi* minicircle variable regions, both within a strain and between strains, allows the classification of *T. cruzi* strains into schizodemes (4, 13), which probably represent naturally occurring clones which have been genetically isolated from each other in nature for long periods (20-22). This classification could serve as an important epidemiological tool for monitoring of specific parasite strains in nature, as well as for understanding the possible role of parasite genetic diversity in the disease syndrome.

In this study, we utilized the guanidine-EDTA-blood-PCR method described above to analyze a large panel of blood samples from chagasic patients which were also analyzed by conventional serologic methods and xenodiagnosis. Comparison of the results obtained with the three different tests indicated that PCR has 100% sensitivity compared with serologic testing and is more sensitive than xenodiagnosis.

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TABLE 1. Results obtained with clinical samples from VL analyzed by serologic,^a xenodiagnostic, and PCR tests

Sample	Clinical diagnosis ^b	Serology ^c	Xenodiagnosis	PCR
VL01	ND ^d	ND	ND	+
VL02	NC	+	-	+
VL03	NC	+	+	+
VL04	NC	+	+	+
VL05	C	+	+	+
VL06	C	+	-	+
VL07	NC	-	ND	+
VL08	NC	+	-	+
VL09	C	+	-	+
VL10	C	+	+	+
VL11	NC	+	-	+
VL12	NC	+	ND	+
VL13	C	+	+	+
VL14	C	+	-	+
VL15	C	+	+	+
VL16	C	+	+	+
VL17	C	+	-	+
VL18	NC	+	+	+
VL19	NC	+	+	+
VL20	C	+	+	+
VL21	C	+	+	+
VL22	C	+	+	+
VL23	C	+	-	+
VL24	C	+	+	+
VL25	NC	-	ND	+
VL26	C	+	+	+
VL27	C	+	-	+
VL28	NC	+	+	+
VL29	NC	+	+	+
VL30	C	+	-	+
VL31	C	+	ND	+
VL32	C	+	+	+
VL33	C	+	-	+
VL34	NC	+	+	+
VL35	NC	±	ND	+
VL36	C	+	+	+
VL37	C	+	+	+
VL38	C	+	+	+
VL39	NC	±	-	+
VL40	C	+	+	+
VL41	C	+	-	+
VL42	C	+	ND	+
VL43	NC	+	-	+
VL44	C	+	+	+
VL45	NC	+	-	+
VL46	NC	-	-	-
VL47	C	+	-	+
VL48	NC	+	ND	+
VL49	NC	+	+	+
VL50	NC	+	-	+
VL51	C	+	+	+
VL52	C	+	-	+
VL53	NC	±	ND	+
VL54	C	+	-	+
VL55	C	+	-	+
VL56	C	+	-	+
VL57	NC	+	-	+

Continued

MATERIALS AND METHODS

Patients. The study population was a subgroup of male and female patients (ages, 13 to 84 years) who are part of a longitudinal study on Chagas' disease morbidity and mortality being conducted by one of us (J.B.P.) in the municipality

TABLE 1—Continued

Sample	Clinical diagnosis ^b	Serology ^c	Xenodiagnosis	PCR
VL58	C	+	-	+
VL59	C	+	+	+
VL60	C	+	+	+
VL61	C	+	+	+
VL62	C	+	-	+
VL63	C	+	-	+
VL64	C	+	+	+
VL65	NC	+	+	+
VL66	C	+	+	+
VL67	NC	+	-	+
VL68	C	+	-	+
VL69	NC	+	+	+
VL70	C	-	-	+
VL71	C	+	-	+
VL72	C	+	+	+
VL73	NC	+	-	+
VL74	NC	+	ND	+
VL75	C	+	+	+
VL76	C	+	+	+
VL77	NC	+	+	+
VL78	NC	+	+	+
VL79	NC	+	+	+
VL80	NC	+	+	+
VL81	C	+	+	+
VL82	C	+	+	+
VL83	NC	+	+	+
VL84	NC	+	+	+
VL85	C	+	-	+
VL86	NC	+	-	+
VL87	NC	+	+	+
VL88	C	+	-	+
VL89	C	+	ND	+
VL90	C	+	-	+
VL91	NC	+	+	+
VL92	NC	±	-	+
VL93	C	+	-	+
VL94	C	+	+	+
VL95	NC	+	+	+
VL96	C	+	+	+

^a Positivity thresholds of serologic tests: IIF, >1:40; complement fixation, >1:5; hemagglutination, >1:40; enzyme-linked immunosorbent assay, >1:40.

^b NC, no cardiomyopathy; C, cardiomyopathy.

^c +, three or four serologic tests positive; ±, IIF and one other test positive; -, three or four serologic tests negative.

^d ND, not done.

of Virgem da Lapa (VL), Minas Gerais, Brazil (see Table 1). All of the patients, except VL01, underwent clinical examinations, conventional electrocardiogram tests, and thoracic X-rays. The clinical diagnosis of cardiomyopathy was confirmed by an abnormal electrocardiogram (15).

Serologic tests. All VL samples were analyzed by four different serologic tests. All four tests, indirect immunofluorescence (IIF) (positive, >1:40 dilution), complement fixation (positive, >1:5 dilution), hemagglutination (positive, >1:40 dilution), and enzyme-linked immunosorbent assay (positive, >1:40 dilution), were kits produced by Bio-Manguinhos (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) and were performed in accordance with the instructions of the manufacturer. IIF was used as the reference test whenever discrepant results were obtained with the other three serologic tests (17, 23).

Xenodiagnosis. Forty fourth-instar nymphs, 20 of *Triatoma infestans* and 20 of *Panstrongylus megistus*, were used for each test. The triatomines were allowed to feed for 30

TABLE 2. Comparison of results for blood samples analyzed by serologic, xenodiagnostic, and PCR tests

Sample type	No. of samples	No. of PCR results	
		Positive	Negative
Clinical diagnosis of Chagas' disease and:			
Serology positive	91	91	0
Serology negative	1	1	0
Serology positive and:			
Xenodiagnosis positive	48	48	0
Xenodiagnosis negative	35	35	0
Serology-negative blood of nonchagasic patients from VL			
	3	2	1
UCLA blood bank donors	18	0	18

min on each patient, and the insects' feces were examined microscopically 45 days after feeding for the presence of *T. cruzi*.

Isolation of DNA for PCR. Blood lysates were prepared by mixing equal volumes of blood and GE buffer (6 M guanidine, 0.2 M EDTA, pH 8.0) and then stored at 4°C. Blood specimens from the University of California at Los Angeles (UCLA) blood bank were used as negative controls for the PCR assay. A 5-ml aliquot of each blood lysate sample was processed as described previously (5). Briefly, 0.5 ml of each of the following solutions was added in the following order: 1 M MgCl₂, 200 mM CuSO₄, 20 mM phenanthroline, and 7.5% H₂O₂. The blood lysates were then incubated at 37°C for 30 min, and the reaction was stopped by addition of 0.5 ml of 58 mM 3-mercaptopropionic acid. After 1,10-phenanthroline-copper ion cleavage of the DNA, a 500-μl aliquot of each blood lysate was extracted with 100 μl of phenol-chloroform (1:1). The supernatants were transferred to a clean tube, and the DNA was precipitated at room temperature by addition of 50 μl of 3 M sodium acetate and 1 ml of ethanol. The DNA pellets were resuspended in 1 ml of water and washed twice with water in Centricon-100 centrifugal filtration units (Amicon) as described by the manufacturer. The DNA was finally resuspended in 200 μl of water. In recent experiments, we have found that the Centricon filtration step can be eliminated by a single extraction of the cleaved blood lysate with a resin (Strataclean resin; Strata-gene) which removes most of the protein prior to precipitation of the DNA (data not shown). All of the PCR amplifications reported in this study, however, were performed with Centricon purification.

PCR. A 70-μl aliquot of the resuspended DNA was PCR amplified by using *T. cruzi*-specific minicircle primers S35 (5'-AAATAATGTACGGG[T/G]GAGATGCATGA-3') and S36 (5'-GGGTTTCGATTGGGGTTGGTGT-3'). The primers were annealed to the four conserved regions of each *T. cruzi* minicircle molecule (4, 19). A 330-bp PCR amplification product which encompassed all of the adjacent variable regions and a small portion of the conserved region (approximately 70 bp) was generated. PCR conditions have been described previously (4, 5). Denaturation, annealing, and elongation steps were performed for a total of 30 cycles, for 1 min each at 94, 60, and 72°C, respectively.

Analysis of 330-bp PCR products. The PCR products were chloroform extracted and ethanol-precipitated, and each

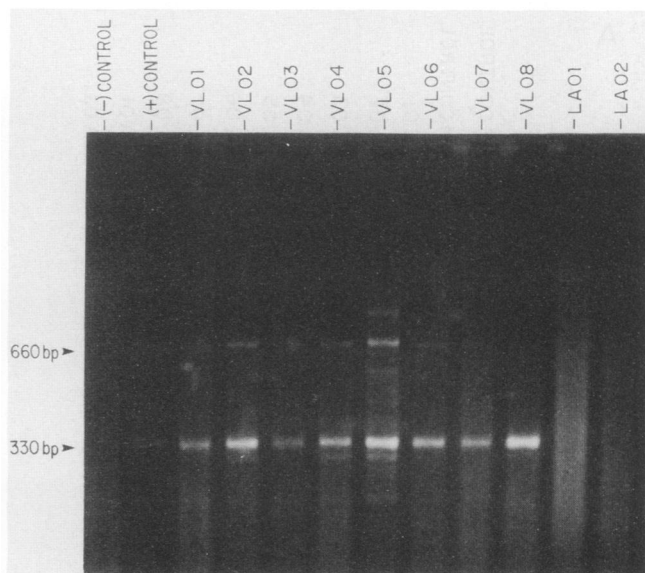


FIG. 1. Representative results of PCR amplification of *T. cruzi* minicircle kDNA present in whole blood lysates of chronic chagasic patients from VL. Blood samples were processed as described in Materials and Methods. The PCR products (one-half of each PCR reaction) were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The 330-bp band in each lane is the expected *T. cruzi*-specific product. The LA01 and LA02 samples are negative control blood specimens obtained from the UCLA blood bank. Negative (-) control lane, no kDNA in PCR; positive (+) control lane, 100 fg of *T. cruzi* kDNA in PCR.

DNA pellet was resuspended in 20 μl of water. Half of each reaction (10 μl) was loaded in a 2% agarose gel, and the gel was blotted onto a Magna NT nylon membrane. The membrane was hybridized with *T. cruzi* minicircle probe S67 (5'-TGGTTTGGGAGGG[C/GG][C/G][T/G]TCAA[A/C]TTT-3'), which hybridizes to the conserved region of *T. cruzi* minicircle molecules (19). The oligonucleotide probe was labeled by tailing with digoxigenin dUTP as described by the manufacturer (Boehringer Mannheim) or by 5'-end kinase treatment with [γ -³²P]ATP. The Southern blots were hybridized at 37°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate-1× Denhardt's solution-0.05% Na PP_i, washed in 6× SSC-0.1% sodium dodecyl sulfate at 37°C for 20 min, and then washed under high-stringency conditions in 3 M tetramethyl ammonium chloride at 65°C for 30 min (4). Detection of the digoxigenin-labeled hybridized probe was performed as described by the manufacturer (Boehringer Mannheim), and detection of the ³²P-labeled probe was performed by exposure of the blot to X-ray film at -80°C.

RESULTS

Blood specimens. Three separate blood specimens were obtained from each patient during the same visit. The blood specimens were used for serologic, xenodiagnostic, and PCR tests. Blood from anonymous donors at the UCLA blood bank and blood from one of us (L.S.) were used as negative controls for the PCR test. Previous studies assessing the prevalence of *T. cruzi* infection among blood donors at a Los Angeles County blood bank indicated that the rate of infected donors is approximately 1 per 1,000 (1, 11); therefore, it is highly probable that the small number of transfusion-

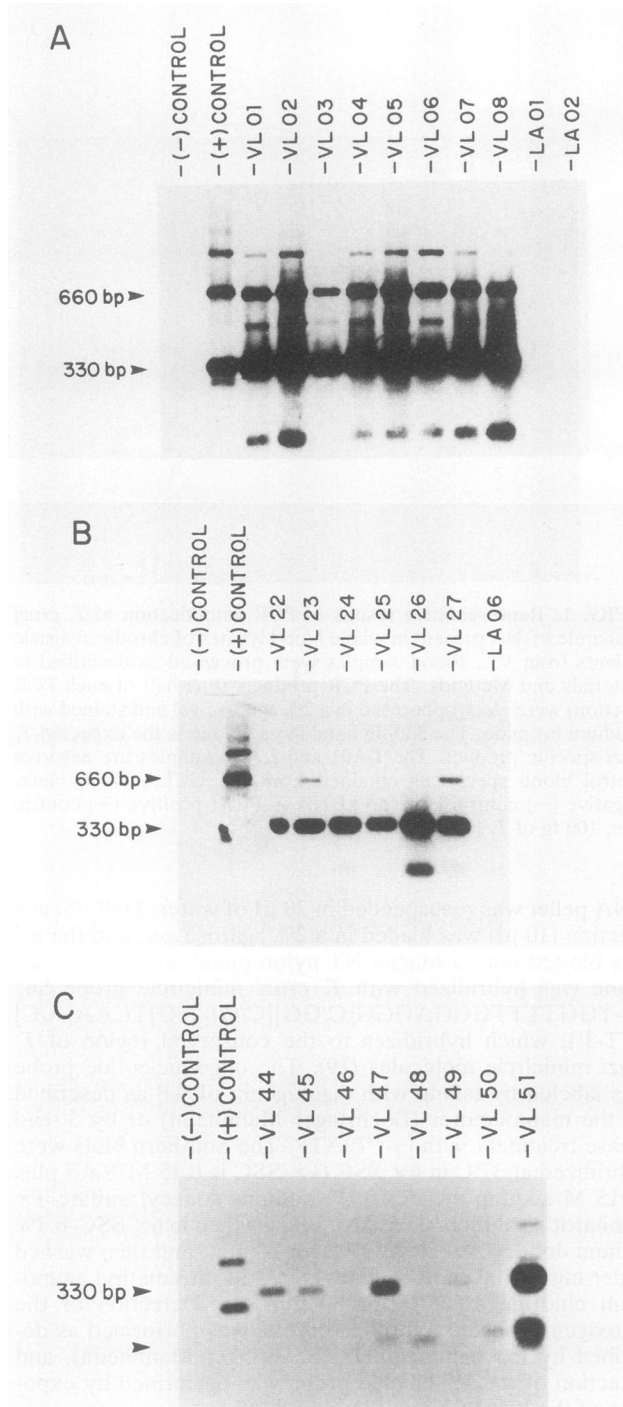


FIG. 2. Representative results of detection of *T. cruzi* minicircle kDNA in chronic chagasic patients by PCR amplification and hybridization. Samples were processed as described in Materials and Methods. The PCR products (one-half of each PCR) were electrophoresed in 2% agarose gels and blotted onto nylon membranes. The blots were hybridized with a digoxigenin-labeled *T. cruzi*-specific minicircle probe (S67). VL lanes, samples from VL (numbers refer to the sample numbers in Table 1); LA lanes, samples from the UCLA blood bank used as negative controls (numbers denote different samples); negative (-) control lanes, no kDNA in PCR; positive (+) control lanes, 100 fg to 1 pg of *T. cruzi* kDNA in PCR. (A) Hybridization results of gel shown in Fig. 1. Sample VL07 was serology negative. (B) Sample VL25 was serology negative. (C) The positive control was diluted 10,000-fold in H₂O to

ready UCLA blood samples we analyzed were indeed negative for *T. cruzi*.

Serologic results. Only 4 of 95 patients from VL had negative serologic results in all four tests (Table 1). However, one of these patients (VL70) had clinical symptoms of chagasic infection. Additionally, four more patients (VL35, VL39, VL53, and VL92) had conflicting serologic results. However, they were considered positive for our analysis because their IIF test results were positive. Whenever a new test for *T. cruzi* has been evaluated, IIF has been the reference test because of its high sensitivity (17, 23).

Xenodiagnosis. A total of 83 serology-positive patients were examined by xenodiagnosis. Only 48 (54%) of these patients were positive by xenodiagnosis (Table 2). This is consistent with previous reports that indicate only 17 to 70% agreement between serologic and xenodiagnostic results (18).

PCR. Figure 1 shows an example of products, obtained after PCR amplification of clinical specimens with the S35 and S36 primer set, electrophoresed in an ethidium bromide-stained agarose gel. There is some variation from sample to sample in the intensity of the background bands and *T. cruzi*-specific bands. Figure 2 shows representative hybridization results obtained for the PCR-amplified samples with the digoxigenin-labeled S67 probe. None of the background bands seen in Fig. 1 gave a signal when the Southern blots were hybridized with the S67 probe (Fig. 2A). Hybridizations with digoxigenin-labeled probes showed the same level of sensitivity as hybridizations with ³²P-labeled probes (data not shown). Identical results were obtained with both types of probes; however, the use of digoxigenin-labeled probes avoids all of the problems and hazards associated with ³²P-labeled probes without compromising the results. The 330-bp band is the expected size of *T. cruzi* PCR-amplified products obtained with the S35 and S36 primer set. The larger PCR products of 660 and 990 bp (Fig. 2) are multiples of the minicircle variable regions and are due to the fourfold repeat structure of the *T. cruzi* minicircles. The nature of the smaller 180-bp band seen in some samples (Fig. 2) is unknown and is being investigated. However, it is important to note that this 180-bp band is *T. cruzi* specific since it appeared only when *T. cruzi* minicircle DNA was present.

Samples from the UCLA blood bank were used as negative controls. Addition of kDNA to several of these control samples prior to amplification indicated that they did not inhibit PCRs. The UCLA blood samples were processed simultaneously with the coded samples from VL to monitor for any cross-contamination problems which may have arisen during the preparation of specimens for PCR. All of these controls proved PCR negative, indicating a lack of contamination in the PCR reagents and during processing of the samples (Fig. 2 and Table 2).

It should be noted that the presence or absence of a 330-bp PCR-generated product in the blots is unambiguous compared with negative controls. The only exceptions were samples VL48 and VL50, which showed no 330-bp PCR product but showed the 180-bp *T. cruzi*-specific PCR prod-

match the signal intensity of the sample lanes. The anomalous (faster) migration of the 330-bp band in the positive control and undiluted VL samples. Sample VL46 was serology negative. Samples VL48 and VL50 exhibited only the 180-bp *T. cruzi*-specific PCR product (arrow).

uct. Therefore, for our analysis these two samples were classified PCR positive. Although several sets of samples did yield less intense 330-bp bands, there is no evidence that this represents any quantitation of the number of parasites present in the blood samples and this variation in intensity could very well be due to variations in sample preparation.

Comparison of serologic, xenodiagnostic, and PCR test results. All serology-positive patients had positive PCR test results, indicating 100% sensitivity of the PCR assay compared with the serologic tests (Table 2). Furthermore, patient VL70, who was diagnosed as having cardiomyopathy characteristic of chronic Chagas' disease but was serology negative, had a positive PCR result. This is not surprising, since it is known that certain chagasic patients remain serology negative throughout their lives (7). In addition, it is important to note that all serology-positive, xenodiagnosis-negative patients had positive PCR test results. This suggests that PCR is more sensitive than xenodiagnosis in the diagnosis and monitoring of chronic chagasic patients. However, of three presumed nonchagasic patients, VL07 (Fig. 2A), VL25 (Fig. 2B), and VL46 (Fig. 2C), two (VL07 and VL25) had positive PCR test results. All of the PCR controls indicated absence of contamination, so it is possible that these two patients are serology-negative chagasic patients showing no cardiomyopathies.

DISCUSSION

PCR amplification of DNA sequences is a highly sensitive technique which is particularly useful for detection of infectious agents that are present at low titers in clinical specimens (9, 10, 14). In the case of chronic Chagas' disease, direct detection of *T. cruzi* cells in peripheral blood is extremely difficult and direct DNA hybridization methods have proved unsuccessful (3). We have shown in this study that PCR amplification of minicircle kDNA sequences appears to be an appropriate technique for detection of *T. cruzi* in chronic chagasic patients. In addition, we have shown that the guanidine-EDTA lysis reagent is applicable to blood sample collection and storage in the field in a tropical environment.

An alternative approach has recently been reported in which *T. cruzi* parasite DNA which was detected in serum samples was used for amplification of a 195-bp *T. cruzi*-specific nuclear repetitive sequence (16). The five acute-stage and seven chronic-stage patients analyzed in this study were hemoculture positive, indicating a fairly high level of parasitemia. It needs to be determined whether this approach is sensitive enough to detect chronic chagasic patients with very low levels of parasitemia who are hemoculture and/or xenodiagnosis negative.

The purpose of our study was to evaluate the performance of PCR amplification of kDNA minicircle sequences in the diagnosis of chronic Chagas' disease by using whole blood specimens from patients with known clinical histories. In particular, we wanted to compare PCR results with results obtained by using xenodiagnosis and classical serologic tests. The panel of seropositive individuals analyzed was composed of chronic chagasic patients with or without cardiomyopathies. Since few serology-negative patients were included in this panel, the specificity of the PCR assay under field conditions could not be determined. Clearly, a larger panel including a greater number of nonchagasic patients must be analyzed. However, we showed previously that the PCR primers used are *T. cruzi* species specific and do not react with the DNA of other kinetoplastids, like

Leishmania sp. and *T. rangeli* (19), or with other infectious agents, like cytomegalovirus, human immunodeficiency virus, and hepatitis B virus (data not shown). In addition, after hybridization with a *T. cruzi*-specific minicircle conserved-region probe (S67 oligonucleotide), all except two of the PCR-positive samples yielded the 330-bp product characteristic of *T. cruzi* minicircles. However, these two samples, VL48 and VL50, showed only the 180-bp *T. cruzi*-specific PCR product. No nonspecific hybridization bands were observed in any sample. These results indicate that none of the hybridization-positive signals was due to cross-reactivity between our oligonucleotides and non-*T. cruzi* DNA sequences.

Our results indicate that the kDNA PCR amplification method has 100% sensitivity compared with serologic testing. Furthermore, the PCR method gave a positive result in all of the cases that were serology positive but xenodiagnosis negative. Clearly, xenodiagnosis lacks sensitivity. These results suggest that PCR could replace xenodiagnosis in the direct parasitological evaluation of chronic chagasic patients. Such an evaluation is necessary for monitoring of drug treatment of chronic chagasic patients and cure determination.

The serologic tests for Chagas' disease have very high sensitivity, from 95 to 100%, in the chronic phase of the disease (7, 17). However, the main problem for blood banks screening for chagasic patients is the variable number of cases reacting around the cutoff level in the present serologic tests (2); therefore, a confirmatory test for the presence of *T. cruzi* parasites in the blood is necessary. The PCR method could serve as the confirmatory test for Chagas' disease in blood banks.

Another advantage of the kDNA PCR amplification method lies in the ability to use the inherent sequence polymorphisms of the amplified minicircle variable regions to classify the parasites as schizodemes or clones (4, 6). This would allow investigation of the question of a correlation of the clinical syndrome and the parasite schizodeme and would also serve as an important epidemiological tool. This work is in progress for the samples from VL.

The DNA isolation procedure we have developed yields total blood DNA as a template, allowing possible coamplification of human genetic loci, for example, the human major histocompatibility complex, in addition to infectious agent-specific DNA sequences. The development of a PCR multiplex system which allows coamplification of both human and pathogen DNA sequences could further our understanding of the interactions between pathogen and host factors and the role these interactions play in disease development.

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