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Polymerase Chain Reaction-Based Detection of *Trypanosoma cruzi* DNA in Serum

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DNAs prepared from chagasic patients' sera were amplified by the polymerase chain reaction using oligonucleotide primers which anneal specifically to a highly repetitive sequence of *Trypanosoma cruzi* nuclear DNA. Samples from both acutely and chronically infected patients yielded positive results by this method. No significant difference was observed when either whole blood or serum samples of the patients were used. These results indicate that serum instead of whole-blood samples could be used for polymerase chain reaction-based detection of *T. cruzi* in field studies without the need of applying any special chemical treatment to the specimens. This would represent a considerable advantage due to the easier handling and transportation of serum as compared with whole-blood samples, especially in tropical climates.

Chagas' disease, which is responsible for considerable morbidity and mortality in Latin America, is caused by a protozoan parasite, *Trypanosoma cruzi*. The diagnosis of this infection is usually performed by microscopic examination of fresh blood samples (1, 5), by hemoculture (9), or by xenodiagnosis (3, 13), as well as by serological methods. However, since an antigen-antibody cross-reactivity phenomenom with *Leishmania* antigens has been reported (4), an unequivocal diagnosis of Chagas' disease, especially in areas where endemic *T. cruzi* and *Leishmania* infections overlap, demands the direct observation of parasites isolated from the patients.

In the early acute stage of the disease, the diagnosis is rather easy because of the high parasitemia levels, which can be detected by direct methods. In contrast, during the chronic stages, the low parasitemia often precludes parasite detection in fresh blood (3).

The polymerase chain reaction (PCR) has been successfully employed in reduviid insects and in mice (8) as well as in the blood of chagasic patients (2, 14) for *T. cruzi* detection. Moser et al. (8) have used the oligonucleotide pair TCZ1 and TCZ2 to amplify *T. cruzi* nuclear DNA (6), whereas Avila et al. (2) have recently reported the amplification of kinetoplast minicircle DNA in blood specimens preserved with guanidine-EDTA.

Since field studies usually involve the transportation of specimens from distant places to the laboratories, where they are often stored for a certain time before examination, we have explored the feasibility of using sera rather than whole blood for diagnosis of *T. cruzi* infection by PCR.

MATERIALS AND METHODS

Parasites. *T. cruzi* epimastigotes of five reference strains (Tulahuen, Y, Colombiana, Sao Felipe, and Berenice) and four Paraguayan *T. cruzi* isolates (MJL, MS, JAG, and JM) were used in these studies. They were grown in liver infusion tryptose medium supplemented with 5% bovine serum and 100 U of penicillin G per ml and were harvested from 2-week-old cultures. Promastigotes of *Leishmania mexicana amazoniensis* GO2 and GO3 were supplied by Y. Hashiguchi (7).

Animals. Three *Cebus apella* monkeys, M131, M114, and M132, were infected with 3×10^5 *T. cruzi* trypomastigotes (Y strain), and blood samples were obtained from them 4 months after infection, when the parasitemia levels were 36, 17, and 14 parasites per ml of blood, respectively, as determined by the direct micromethod described by Freilij et al. (5) and modified by Arias and Ferro (1).

Hemoculture. Blood samples (5 to 7 ml) were taken from the patients and collected on EDTA. The samples were centrifuged at $2,000 \times g$ for 10 min, and the plasma was replaced by equal volume of liver infusion tryptose medium and incubated at 28°C for 6 months. The cultures were regularly examined by direct microscopic observation, starting from day 30 (9).

Isolation of DNA from blood and sera. Blood samples from monkeys were collected both with and without EDTA. DNA was isolated from 100 μl of either monkeys' blood or serum and from patients' serum samples. All of the samples were brought up to 300 μl with pure distilled water to which 50 μl of 30% Sarkosyl and 1.5 μl of 20-mg/ml proteinase K were added and incubated at 60°C for 1 h. Then, 30 μl of 10% sodium dodecyl sulfate was added, and the samples were mixed. The samples were phenol extracted, and 20 μg of glycogen was added before ethanol precipitation. DNA pellets obtained from patients' sera were suspended in 50 μl of water, whereas those obtained from monkeys' blood and parasite cultures were suspended in 100 μl of water. The same procedure was followed for DNA isolation from cultured parasites.

PCR. PCR was performed in 100-μl reaction mixtures containing 1/10 of the total DNA isolated from 100 μl of either whole blood or sera, 250 μM (each) deoxynucleoside triphosphates, 100 pmol of each primer (TCZ1 and TCZ2 [8]), and 2 U of Tth DNA polymerase (TOYOBO Co. Ltd., Osaka, Japan). The reaction mixtures were overlaid with 75 μl of mineral oil and subjected to 25 cycles of amplification in a thermal sequencer (TSR-300; Iwaki Co. Ltd). The temperature profile for denaturation, primer annealing, and

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primer extension was 95°C for 60 s (with a longer initial time of 300 s at 95°C), 53°C for 90 s, and 73°C for 120 s, respectively (with a final incubation at 73°C for 300 s to extend the annealed primers). One-tenth of the reaction products were electrophoresed on a gel containing 2.5% NuSieve GTG agarose (FMC Bioproducts) and 0.5% Agarose NA (Pharmacia) and visualized after ethidium bromide staining.

In order to keep track of potential cross-contaminations, internal controls were always run in each step. Two serum samples from healthy individuals were included in every DNA isolation batch, whereas in each round of PCR amplification, a stored DNA sample previously isolated from an uninfected person was employed besides the two control DNA preparations of the batch. A reaction mixture in which DNA was omitted was also included in each set of reactions.

Southern blot hybridization. For Southern blot hybridization analysis, PCR products were electrophoresed on 3% agarose gel, stained with ethidium bromide, and transferred onto a Hybond-N^+ membrane (Amersham). The membrane was hybridized with a probe consisting of the 188-bp DNA fragment obtained by TCZ-primed amplification of *T. cruzi* DNA and cloned in pUC18. This probe (checked by sequencing) was labeled with horseradish peroxidase by using the enhanced chemiluminescence gene detection system (Amersham International plc). Hybridization and washing were performed at 42°C. The detection of horseradish peroxidase-labeled probes was carried out by the peroxidase-catalized oxidation of luminol and subsequent enhanced chemiluminescence (15). The emitted light was detected on X-ray film by 1-h exposure at room temperature.

RESULTS

Detection of Paraguayan *T. cruzi* isolates by PCR. It was necessary to make sure that the TCZ primers could promote the amplification of DNA of *T. cruzi* isolates obtained from patients in Paraguay. Moser et al. (8) had already reported that the TCZ oligonucleotides can prime the amplification of DNA from *T. cruzi* strains from widely separated regions in Latin America but failed to amplify DNA of some other species of the *Trypanosomatidae* family. Since overlapping areas in which *T. cruzi* and *Leishmania* strains are endemic exist in Paraguay (4), it was also important to corroborate that *Leishmania* DNA is not amplified. In fact, we have found that the TCZ primers amplified DNA from all Paraguayan *T. cruzi* isolates tested (Fig. 1, lanes 6, 7, 8, and 9), yielding products of the same molecular weight as those of the reference strains (lanes 1, 2, 3, 4, and 5), but failed to amplify *Leishmania* DNA.

Detection of *T. cruzi* DNA in serum by PCR. To test the possibility of detecting *T. cruzi* DNA in sera, blood samples were drawn from chronically *T. cruzi*-infected *C. apella* monkeys, M131, M114, and M132, whose parasitemia values were determined by the direct micromethod (1, 5). Monkeys were chosen for these studies because the course of *T. cruzi* infection in these animals is similar to that in humans, and their parasitemia in the chronic stage is also very low (11, 12). No consistent difference was noticed in the intensity of the ethidium bromide-stained DNA bands after electrophoresis when either whole blood or serum was used as the source of DNA (Fig. 2). Since the concentrations of parasites in the original samples were known, it was possible to estimate the amount of DNA contained in each reaction mixture, namely, 0.72, 0.34, and 0.28 parasite equivalents, respectively. Therefore, we can assume that if sheared DNA is used as template for the PCRs, about one-fourth of a parasite could be readily detected by this procedure.

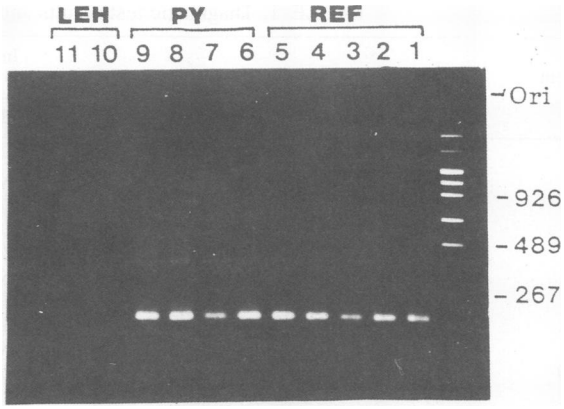

FIG. 1. Amplification of DNA from Paraguayan *T. cruzi* isolates using the TCZ1 and TCZ2 primers. Lanes 1 through 5, PCR products of the reference strains (Tulahuen, Y, Colombiana, SF, and BE, respectively); lanes 6 through 9, PCR products of the Paraguayan isolates (MJ1, MS, JAG, and JM, respectively); lanes 10 and 11, PCR products of the *Leishmania* strains GO2 and GO3, respectively. All the reactions were performed using DNA equivalent to 100 parasites. The reaction products (10 μl) were resolved on a 3% agarose gel and visualized by ethidium bromide fluorescence. Sizes are given on the right in base pairs.

Amplification of *T. cruzi* DNA in the sera of chagasic patients. Serum samples from five acute and seven chronic patients were examined by PCR (Table 1). Every patient whose DNA was chosen for the PCR amplifications was serologically positive for Chagas' disease, and *T. cruzi* parasites were isolated through hemoculture from all of them except for patient 3, who had a positive direct parasitemia

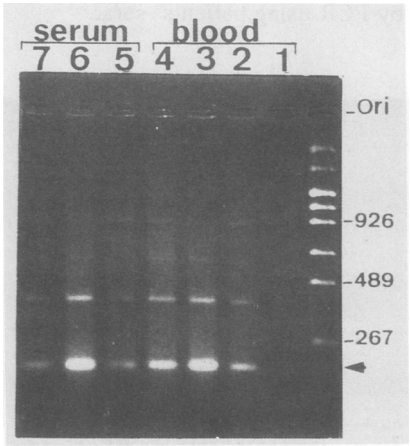

FIG. 2. Amplification of *T. cruzi* DNA in *C. apella* serum samples using the TCZ1 and TCZ2 primers. The PCRs were performed with 1/10 of the DNA isolated from 100 μl of the whole blood (lanes 1 to 4) or serum samples (lanes 5 to 7). One-tenth of each reaction mixture was analyzed by electrophoresis on a 3% agarose gel. Lanes 2 through 4 and 5 through 7 contain the amplification products of DNA from *T. cruzi*-infected monkeys M131, M114, and M132, with parasitemia values of 36, 17, and 14 parasites per ml, respectively. Lane 1 contains the amplification products of DNA from the blood of an uninfected monkey, M121. The gel was stained with ethidium bromide and visualized by UV light. Sizes are given on the right in base pairs. The arrow indicates the 188-bp amplification product.

TABLE 1. Diagnostic test results and clinical symptoms of *T. cruzi* PCR-positive patients

Patient no.	Age (yr)	Clinical symptom	Indirect immunofluorescence titer		ELISA titer[a]		Hemoculture (days)[b]	Direct parasitemia (no. of parasites/ml)
			IgM	IgG	IgM	IgG		
1	12	Romaña's sign[c]	1:160	1:160	1.731	1:10	45	140
2	NB[d]	Congenital	N[e]	1:160	0.270	1:160	40	36
3	23	Romaña's sign	1:160	1:40	1.745	1:20	N	15
4	12	Romaña's sign	1:160	1:80	1.994	1:10	140	N
5	14	Romaña's sign	1:80	1:40	1.460	1:10	140	N
6	65	Cardiomyopathy	N	1:160	0.291	1:80	90	ND
7	45	Cardiomyopathy	1:160	1:160	1.810	1:320	120	ND
8	50	Cardiomyopathy	1:320	1:80	2.840	1:80	134	ND
9	11	Cardiomyopathy	N	1:320	0.287	1:160	138	ND
10	15	Cardiomyopathy	1:20	1:160	1.047	1:320	70	ND
11	59	Cardiomyopathy	1:20	1:640	0.956	1:640	60	ND
12	30	Megacolon	N	1:80	0.284	1:80	180	ND
13	25	None (negative control)	N	N	0.290	N	N	N

[a] ELISA, enzyme-linked immunosorbent assay. IgM values are optical density. The optical density at 405 nm is 0.286 for the negative control.
[b] Time required for detection of parasites in blood cultures. ND, not done.
[c] Romaña's sign is inflammatory swelling of the eyelid, which is a common sign of the acute phase.
[d] NB, newborn without clinical manifestation (congenital transmission).
[e] N, negative.

but negative hemoculture. These facts unequivocally confirm *T. cruzi* infection. Moreover, all of the chronic patients exhibited at least one of the three most common pathologies of Chagas' disease, i.e., megaesophagus, megacolon, and cardiomyopathy. PCRs were performed on one-fifth of the DNA samples isolated from 100 μl of the sera of the chagasic patients. Figure 3 shows the electrophoretic pattern of the PCR products. All of the patients yielded positive results, although some of them had very low levels of circulating parasites, as revealed by the failure of detection by observation of fresh blood samples but successful parasite isolation by hemoculture. These results suggest that at least a small amount of parasite DNA was present in the sera. Therefore, direct diagnosis of *T. cruzi* infection could be performed by PCR using patients' sera.

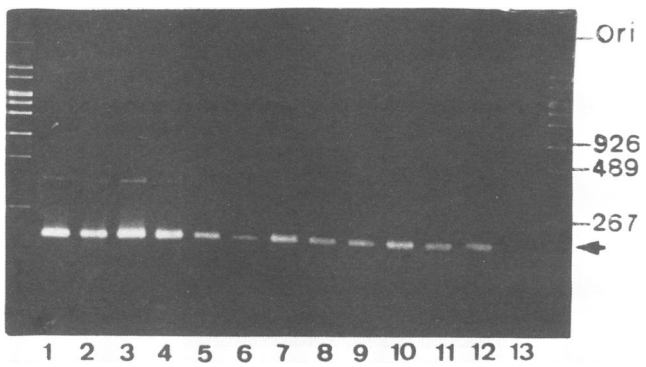

FIG. 3. PCR-based detection of *T. cruzi* DNA in the sera of acute and chronic chagasic patients. The amplifications were performed on one-fifth of the DNA extracted from 100 μl of the serum samples. One-tenth of each reaction mixture was analyzed by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining. Lanes 1 through 3, samples from acute patients with positive direct parasitemia; lanes 4 and 5, samples from acute patients with positive hemoculture; lanes 6 through 12, samples from chronic patients with positive hemoculture; lane 13, sample from an uninfected individual. Sizes are given on the right in base pairs. The arrow indicates the 188-bp amplification product.

PCR-based detection of *T. cruzi* in congenital Chagas' disease. Infants born to seropositive chronic chagasic mothers were examined for the congenital transmission of the disease. Both mothers and newborn babies were checked for the presence of parasites in their bloodstreams by direct microscopic observation and by hemoculture. At the same time, DNA was isolated from their serum samples and amplified by PCR (Fig. 4). The PCR products were visualized both by electrophoresis followed by ethidium bromide staining and by Southern blot hybridization.

Although in some cases no amplification band was visible after ethidium bromide staining of electrophoresed DNA (Fig. 4A, lanes 1, 2, 5, and 10), amplified products were detected by Southern blot hybridization (Fig. 4B). It is interesting to notice that in both cases of congenital Chagas' disease detected in babies I and II, parasitological methods yielded negative results on the very day of birth (Fig. 4A, lanes 1 and 5, respectively), yet the babies were positive by the PCR-labeled probe technique, which indicates that they indeed carried parasites in their bloodstreams. Treatment of these two babies was started when they showed parasitemia by direct microscopic examination of blood at the ages of 3 and 5 months, respectively, when their PCR tests were strongly positive (Fig. 4A, lanes 3 and 6). As for baby III (lane 8), treatment was initiated right after birth.

Individuals who were positive by direct parasitemia showed the strongest bands (lanes 3, 6, 8, and 11), whereas both the seropositive chronic patients (lanes 4, 7, 9, 12, and 13) and the serologically negative patients (lanes 14 and 15) were negative by PCR. The last two groups were negative also by parasitological methods.

DISCUSSION

In countries affected by Chagas' disease it is a common practice to collect samples in distant areas in which the disease is endemic and to remit them to the diagnostic laboratories, where they are often stored for a certain time before examination. Therefore, it is of great interest to design adequate methods for safe transportation of the specimens in order to reduce the risk of deterioration.

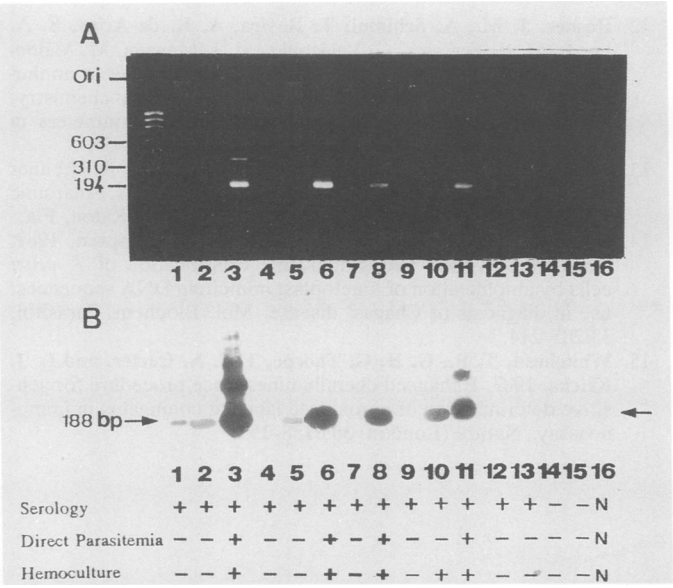

FIG. 4. Detection of *T. cruzi* infection in newborn babies by PCR amplification and hybridization. Agarose gel electrophoresis (A) and Southern blot hybridization analysis (B) of amplified DNA from serum samples. Amplified products of *T. cruzi* DNA were detected in samples from newborn babies. Lanes 1 to 3, samples from baby I on the day of birth and 1 and 3 months later, respectively; lanes 5 and 6, samples from baby II on the day of birth and 5 months later; lane 8, sample from baby III on the day of birth; lanes 4, 7, and 9, samples taken on the day of birth from the chronic chagasic mothers of babies I, II, and III, respectively; lanes 10 and 11, samples from acute patients (positive controls); lanes 12 to 15, samples from patients with chronic Chagas' disease (lanes 12 and 13) and healthy individuals (lanes 14 and 15) (negative controls); lane 16, no DNA in the reaction mixture for PCR amplification.

Recently, Avila et al. (2) reported an interesting method for preservation and transportation of blood specimens to the laboratories for testing of *T. cruzi* infection by PCR. However, serum preparation is a routine task in most rural health care centers, for which reason serum could be a plausible alternative to whole blood for the PCR test.

When PCR was performed using both whole blood and serum from infected monkeys, no remarkable difference was noticed in the intensity of the amplification bands. Therefore, since stocked sera of both chagasic and nonchagasic patients, which had been saved for further serological studies, were at our disposal, we were able to test the possibility of using serum rather than whole blood for a PCR-based diagnosis of *T. cruzi* infection in humans. Fortunately, a high consistency was observed upon comparison of all the results; i.e., samples which were positive by direct parasitemia and/or hemoculture were also positive by PCR.

Although there is a potentially high risk of contamination of the samples with *T. cruzi* genomic sequences during processing, apparently this has not occurred in our case, as revealed by the results obtained with a number of serum samples from our stock, which were from individuals who were negative for *T. cruzi* infection by direct parasitemia, hemoculture, and serology (data not shown). The absence of false-positive PCR results in stocked sera could be easily accounted for by the fact that DNA isolation had not been performed in our laboratory until the start of the PCR works. Moreover, the PCRs were performed in a different laboratory compartment to avoid carryover of amplified sequences. Actually, parasite cultures are routinely performed in a different laboratory with completely separate materials.

The high sensitivity and specificity of the PCR-based diagnosis of *T. cruzi* infection using the TCZ primers make it an excellent candidate for the follow-up of a chemotherapeutic treatment of acute chagasic patients, and the method could also be used to assess parasite clearance from blood. This method would also have a tremendous potential for diagnosis in cases of patient immunodepression and for the study of congenital transmission of Chagas' disease. Infants born to seropositive mothers usually carry anti-*T. cruzi* immunoglobulin G (IgG) antibodies, in which case serological methods are not reliable (10). Although apparently direct parasitemia and hemoculture fail to detect parasites in some newborn babies, the combination of PCR with labeled-probe hybridization could be used for the diagnosis of congenital Chagas' disease. In fact, studies on the congenital transmission of this disease are fairly advanced in our laboratory, with very encouraging results. We have found that of 33 seropositive mothers who were negative by parasitological methods, 8 were positive by PCR but without proven congenital transmission of the disease to their offspring (data not shown). Although this work describes a preliminary evaluation of the use of PCR for the diagnosis of Chagas' disease in serum samples, the results of the study of the congenital transmission show a high consistency between PCR results and parasitemia.

Finally, the detection of amplified *T. cruzi* DNA in sera suggests an active parasite turnover and shedding of its DNA into the bloodstreams of the infected patients. The physiological shearing of this DNA in the bloodstream would allow the distribution of the 195-bp repetitive element in sera which would be very important for a PCR-based diagnosis of *T. cruzi* infection.

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