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Detection of *Trypanosoma cruzi* by DNA Amplification Using the Polymerase Chain Reaction

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The polymerase chain reaction was used to amplify a 188-base pair (bp) segment of the repetitive 195-bp nuclear DNA sequence of *Trypanosoma cruzi* that is the most abundant sequence in this organism. The reaction amplified this repetitive element in four *T. cruzi* isolates from widely separated geographic regions. No amplification of the 188-bp fragment occurred when DNAs extracted from *Leishmania* spp., African trypanosomes, or blood samples from mice and humans were used. Amplification of one-half of the DNA from a single *T. cruzi* parasite produced an amount of the 188-bp element that was readily visible in a gel stained with ethidium bromide. Hybridization of a radiolabeled probe to membrane-bound amplification products increased the sensitivity to a level at which 1/200 of the DNA in a single parasite could be detected. *T. cruzi* DNA was readily detected in DNA extracted from the abdominal contents of infected insect vectors reared in the laboratory. No parasite DNA was detected in the blood samples of two individuals known to be infected with *T. cruzi*, possibly because in such patients the numbers of circulating parasites are extremely low or because parasitemias are intermittent. These results represent a considerable increase in sensitivity over previously reported methods for the detection of *T. cruzi* infections. Polymerase chain reaction amplification can be used to evaluate large numbers of samples in a single day and thus should be useful in large-scale studies of the prevalence of *T. cruzi* in both insect vectors and mammalian hosts.

American trypanosomiasis, or Chagas' disease, is a zoonosis caused by the protozoan hemoflagellate *Trypanosoma cruzi* (3). An estimated 24.7 million people are infected with *T. cruzi* in Latin America, where Chagas' disease is a major cause of morbidity and death in countries endemic for *T. cruzi* (29). In the initial phase of *T. cruzi* infection, which lasts 4 to 6 weeks, large numbers of parasites circulate in the bloodstream. During this period, the diagnosis is based on detection of these organisms by microscopic examination of fresh blood samples. In contrast, chronic Chagas' disease, which follows the resolution of the acute infection, is characterized by levels of circulating parasites far below the threshold for microscopic detection and by high titers of antibodies directed against *T. cruzi* antigens. Diagnosis of chronic Chagas' disease is based primarily on the detection of these antibodies by serologic tests, such as complement fixation and indirect immunofluorescence assays. Generally these assays are quite sensitive, but the occurrence of false-positive reactions has been a persistent problem (4). *T. cruzi* infections in seropositive individuals can be confirmed by xenodiagnosis (21). This laborious procedure involves feeding blood from a patient to laboratory-reared reduviid bugs, the natural vector of *T. cruzi*, and subsequent dissection of the insects and microscopic examination of their intestines for parasites. Xenodiagnosis is highly specific but its sensitivity is limited, since parasites are detected in at most 50% of individuals known to be infected. Thus there is a need for a highly sensitive and specific parasitologic method for confirming chronic *T. cruzi* infections in humans, and such an assay could also have wide applicability in quantifying *T. cruzi* infections in laboratory mammals and insect vectors.

Recently, the polymerase chain reaction (PCR), a technique for DNA amplification in vitro (22, 27), has been used in the molecular diagnosis of some genetic diseases and in the detection of infectious agents (8, 16, 17, 24). In this report, we describe the use of PCR to detect *T. cruzi* in the insect vector and in mammalian hosts. A family of 195-base-pair (bp) DNA repeats that constitutes approximately 9% of the nuclear DNA in *T. cruzi* (10, 19, 32) was amplified in vitro by PCR and detected visually by ethidium bromide staining after electrophoresis or by hybridization to a radiolabeled probe after blotting to a membrane. The level of sensitivity achieved by our assay is many times greater than that achieved previously with DNA probes (2, 10). Since a large number of samples can be evaluated in a day by this detection procedure, it should be useful in large-scale studies of the prevalence of *T. cruzi* in vectors and mammalian hosts.

MATERIALS AND METHODS

Parasites. Epimastigotes of Tulahuén (T. Pizzi, Ph.D. thesis, Universidad de Chile, Santiago, 1957), Y (30), and Corpus Christi (33) strains and the Sylvio X-10/4 clone of *T. cruzi* (31) and promastigotes of *Leishmania mexicana mexicana* and *Leishmania major* (Morton and Friedlin strains; F. A. Neva, unpublished data) were produced in liquid culture as described previously (14, 15). Bloodstream trypomastigotes of *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense*, *Trypanosoma brucei gambiense*, and *Trypanosoma congolense* (6, 20, 23) were produced in Sprague-Dawley rats and isolated on Percoll gradients (11).

The Tulahuén strain of *T. cruzi* was maintained in BALB/c mice, and the infection was passed every 7 to 15 days by intraperitoneal injection of heparinized infected blood into methoxyflurane-anesthetized mice. Reduviid bugs were infected with *T. cruzi* by allowing them to feed directly on mice with high parasitemias (>4 × 10^5 trypomastigotes per ml). The insects were infected as first- or second-instar nymphs and thereafter fed only on acutely parasitized mice. Urine

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was collected from individual insects after blood meals, as described elsewhere (L. V. Kirchhoff and D. F. Hoft, submitted for publication).

Preparation of DNA samples. Samples of mouse and human blood were collected in tubes containing EDTA (final concentration, 10 mM) to prevent coagulation. The samples were processed immediately or stored at −20°C, and DNA was extracted from 100-μl (mouse blood) or 2-ml samples (human blood). For isolation of DNA from reduviid bugs, their abdominal contents were extruded into a microcentrifuge tube and mashed with an applicator stick. DNA was extracted from a 100-μl sample of this material from each insect. Five volumes of lysis buffer (10 mM Tris hydrochloride [pH 7.6], 10 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, 300 μg of proteinase K per ml) was added to these blood and insect samples and incubated for 2 h at 55°C. Samples were then extracted twice with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), and nucleic acids were precipitated by the addition of 1/10 of a volume of 3 M sodium acetate (pH 5.5), 20 μg of glycogen (Boehringer Mannheim Biochemicals), and 2 volumes of ethanol. After centrifugation in a microcentrifuge for 15 min, the pellet was rinsed with 70% ethanol, air dried, and suspended in 100 μl of water. For urine samples from insects, 50 μl was simply diluted 10-fold in water and heated to 100°C for 10 min, and 1 μl was added to the amplification reaction.

Cloning and sequencing of the *T. cruzi* major repetitive element. *Sac*I-digested DNAs (10 μg) from Y and Tulahuén strains of *T. cruzi* were fractionated electrophoretically through a 2% NuSieve GTG agarose gel (FMC BioProducts). After the gel was stained with ethidium bromide, a prominent band of approximately 200 bp, containing fragments of the major repetitive element, was excised. The DNA was purified from the low-melting-point agarose on a NACS cartridge (Bethesda Research Laboratories, Inc.) and cloned into the unique *Sac*I site of the plasmid pIBI30 (International Biotechnologies). Nucleotide sequences of cloned fragments were determined by using a modification of the Sanger chain termination method (12, 28).

Oligonucleotides. The primers used for the PCR amplification, designated TCZ1 and TCZ2, were made on a DNA synthesizer (Beckman Instruments, Inc.). The sequence of TCZ1 is dCGAGCTCTTGCCCACACGGGTGCT; the sequence of TCZ2 is dCCTCCAAGCAGCGGATAGTTC AGG.

PCR amplification. PCR amplifications were performed in 50-μl reaction mixtures containing 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each of the four deoxynucleoside triphosphates, 10 μM each of primers TCZ1 and TCZ2 (see Fig. 1), and 1.25 U of *Taq* DNA polymerase (The Perkin-Elmer-Cetus Corp.). Template DNA was denatured for 10 min at 94°C prior to its addition. In the assays for *T. cruzi* DNA in insect abdominal contents and in mouse blood, approximately 0.5 μg of phenol-extracted DNA from these sources was used as the template. Reaction mixtures were overlaid with paraffin oil to prevent evaporation and subjected to 25 cycles of amplification in a programmable thermal cycler (Perkin-Elmer-Cetus). During each cycle, the samples were incubated at 94°C for 30 s (to denature the template), cooled to 55°C for 30 s and incubated at that temperature for 60 s (to anneal the primers), and heated to 72°C for 30 s and incubated at the same temperature for 90 s (to extend the annealed primers). The temperature was then returned to 94°C during a 30-s period to initiate the next cycle. At the end of the last cycle, samples were incubated an additional 3 min at 72°C and then cooled for 10 min to room temperature. Ten microliters of each sample (one-fifth of the total volume) was subjected to electrophoretic fractionation in a 2.5% NuSieve GTG agarose gel (FMC Corp.) containing 1 μg of ethidium bromide per ml.

Slot-blot analysis. The amplification reaction products were denatured and applied through a slot-blot apparatus (Bethesda Research Laboratories) to a charge-modified nylon membrane (BioTrace RP; Gelman Sciences, Inc.) (1). Prehybridization, hybridization, and washing steps were performed according to protocols recommended by Gelman Sciences. The restriction fragment used as a probe was radiolabeled with ^{32}P by the random primer method (9). Autoradiography was on XRP-1 film (Eastman Kodak Co.) at −70°C with two Cronex intensifying screens for 24 h.

RESULTS

Cloning and sequence analysis of *T. cruzi* major repeat elements. As an initial step in the development of a PCR-based detection method, we cloned three copies of the repetitive element into pIBI30 (pTCR-Y5 and pTCR-Y6 from Y strain and pTCR-T8 from Tulahuén strain) and determined their sequences. Figure 1 shows a comparison of these sequences with five other clones of the repeat that were reported previously. The sequences vary only in a small number of positions, indicating clearly that we have cloned repetitive elements that belong to the same family as those reported earlier.

Specificity of the PCR-based detection method for *T. cruzi*. The primer pair TCZ1 and TCZ2 (Fig. 1) should prime amplification of a 188-bp sequence that lies within the 195-bp repetitive element. The A-T-rich sequence (AATTTCG, positions 189 to 195) was excluded from the primers in an effort to reduce their potential nonspecific annealing to A-T-rich regions of parasite, insect, or mammalian DNAs that are also present in the amplification reactions.

To establish the sensitivity and specificity of amplification primed by TCZ1 and TCZ2, we used genomic DNAs from various sources as templates for the reaction. When DNAs from the Y, Tulahuén, Corpus Christi, and Sylvio X-10/4 isolates of *T. cruzi* were used, the expected 188-bp bands were clearly evident after electrophoretic separation of the reaction products and ethidium bromide staining (Fig. 2, lanes 1 to 4). These results suggest that the 195-bp element is universally present in *T. cruzi*, since these four isolates were obtained from patients in widely separated areas (southern Brazil, Chile, Texas, and northern Brazil, respectively). In contrast, when DNAs from sources other than *T. cruzi* were used in the reaction, no amplification of the 188-bp segment was detected. Specifically, TCZ1 and TCZ2 do not prime amplification of sequences in *Leishmania* spp. (*L. mexicana mexicana* and *L. major*, lanes 5 and 6, respectively), which are members of the same family (*Trypanosomatidae*) and infect humans in areas endemic for *T. cruzi* (18). Likewise, they do not prime amplification of DNA from the African trypanosomes, *T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, and *T. congolense* (lanes 7 to 10, respectively), which are also closely related phylogenetically to *T. cruzi*. In addition, mammalian hosts of *T. cruzi*, such as mice or humans, do not have DNA sequences that are amplified to any significant degree with these primers (lanes 11 and 12). However, when greater quantities of mammalian DNA are present in the reaction, several minor bands appear which are larger than the 188-bp band of *T. cruzi* DNA, as discussed below.

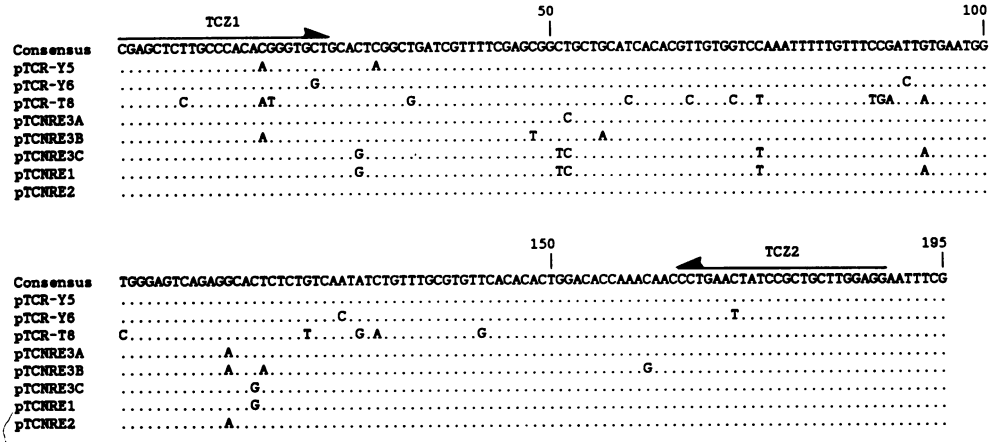

FIG. 1. Nucleotide sequences of eight cloned copies of the *T. cruzi* repetitive element and a derived consensus sequence. Dots indicate positions of identity. The sequences of pTCR-Y5 and pTCR-Y6 from Y strain and pTCR-T8 from Tulahuén strain were determined by our laboratory. The remaining sequences are taken from reference 10. The sequences of oligonucleotide primers TCZ1 and TCZ2 used for PCR amplification (→) are indicated. TCZ1 contains the sequence shown, and TCZ2 has a sequence complementary to that shown. The *Sac*I site (GAGCTC) used to clone the repeats is located at position 2.

Detection of *T. cruzi* in an insect vector by using PCR amplification. *T. cruzi* infection rates in insect vector populations are usually determined by microscopic detection of parasites in the abdominal contents of individual reduviid bugs (25). To develop an alternative laboratory method, we tested the ability of PCR amplification to detect the presence of *T. cruzi* in reduviid bugs reared in captivity. Since urine from infected bugs often contains parasites, our first approach was to collect urine samples for analysis. Figure 3 (left) shows the results obtained with urine samples from an uninfected bug (lane 1) and three infected bugs (lanes 2 to 4). No detectable bands were obtained when DNA from the urine of the uninfected insect was added to the reaction, but 188-bp fragments were clearly evident when DNAs from the urine samples of the *T. cruzi*-infected bugs were used.

Obtaining urine samples from insects outside the laboratory is difficult, so DNA was also isolated from the abdominal contents of the same four bugs. The results of PCR amplification of this material are shown in Fig. 3 (right). Again, no 188-bp fragments were present when abdominal DNA of the uninfected bug was used in the reaction (lane 1), while a prominent band of this size was obtained with DNA from each of the infected bugs (lanes 2 to 4).

Detection of parasites in mammalian blood. To assess the ability of the PCR technique to detect *T. cruzi* in mammalian blood, 100-μl samples of mouse and human blood containing 8,000, 800, 80, 8, and 0 parasites were prepared and analyzed as described in the legend to Fig. 4. The amplified 188-bp *T. cruzi* fragment was clearly detectable in all of the parasite-containing mouse blood samples (lanes 1 to 4), but it was not present in the uninfected sample (lane 5). Identical results were obtained with the human blood samples (results not shown). Several minor bands also appeared, especially in the reaction products obtained with the samples containing few or no parasites (lanes 3 to 5). Presumably these products are the result of annealing and extension of primers to mouse DNA sequences that have some degree of similarity to the primer sequences. Since the DNAs in these bands are larger than 188 bp, they do not interfere with the recognition of the *T. cruzi* band. However, experiments in which constant

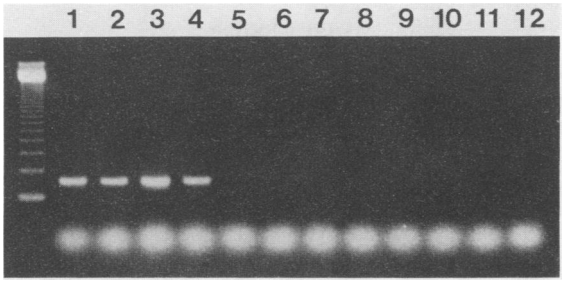

FIG. 2. Photograph of an agarose gel stained with ethidium bromide, showing the species specificity of the *T. cruzi* detection assay. PCR amplifications were performed on 20 pg of DNA (equivalent to DNA in about 60 parasites) from the Y, Corpus Christi, and Tulahuén strains and Sylvio X-10/4 clone of *T. cruzi* (lanes 1 to 4, respectively). Amplification reactions were also conducted on 20 pg of DNAs from *L. mexicana mexicana* (Morton strain), *L. major* (Freidlin strain), *T. congolense*, *T. brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesiense*, human, and mouse (lanes 5 to 12, respectively). A 10-μl sample of the reaction mixture (one-fifth of the total) was applied to each lane. Excess primers appear as a diffuse band in the lower part of each lane. The leftmost lane shows a ladder of 123-bp fragments (Bethesda Research Laboratories).

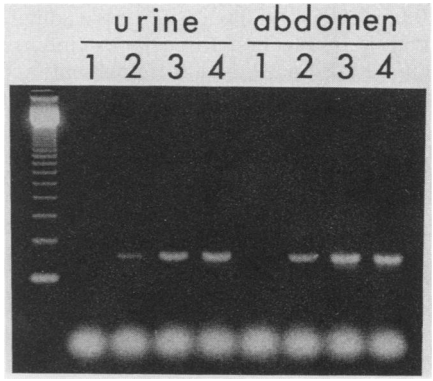

FIG. 3. Photograph of an agarose gel stained with ethidium bromide containing the amplification products of DNAs obtained from the urine or abdominal contents of four reduviid bugs, designated 1 to 4. Bug 1 was not infected, and bugs 2 to 4 were infected with *T. cruzi*.

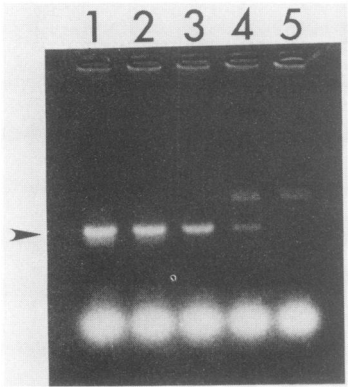

FIG. 4. Photograph of an agarose gel stained with ethidium bromide, demonstrating the detection of *T. cruzi* in mouse blood. Blood from a *T. cruzi*-infected mouse (80,000 parasites per ml) was diluted serially with uninfected mouse blood to prepare 100-μl samples containing 8,000, 800, 80, 8, and 0 parasites (lanes 1 to 5, respectively). Approximately 1/10 of the DNA extracted from each sample was used for PCR amplification. After 25 cycles, one-fifth of each reaction mixture was applied to the agarose gel for electrophoretic analysis. The 188-bp amplification product (▶) is shown.

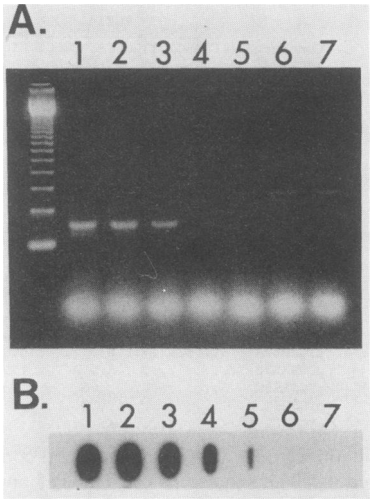

FIG. 5. Detection of the 188-bp *T. cruzi* segment after PCR amplification of various amounts of *T. cruzi* DNA mixed with constant amounts of mouse DNA. DNA was extracted from *T. cruzi*-infected mouse blood (50,000 parasites per ml) and serially diluted 10-fold with DNA extracted similarly from uninfected mouse blood. Amplification reactions were performed on samples containing the DNA from 10 μl of mouse blood, and one-fifth of the reaction products were separated electrophoretically prior to ethidium bromide staining (A). Alternatively, two-fifths of the reaction products were alkali denatured and applied onto a charge-modified nylon membrane, using a slot-blot apparatus (B). After amplification to the nylon membrane, the reaction products were probed with a ^{32}P-labeled *Apa*LI-*Mae*III restriction fragment from the cloned *T. cruzi* repetitive element in plasmid pTCR-Y6 (positions 24 to 121, Fig. 1). This 98-bp fragment, which lies between the two oligonucleotide-priming sites, was used to detect the amplified 188-bp sequence without prior removal of excess PCR primers. Lanes 1 to 7 show the results obtained with these two procedures when DNAs equivalent to blood samples containing 500, 50, 5, 0.5, 0.05, 0.005, and 0 parasite equivalents, respectively, were added to reaction mixtures.

amounts of parasite DNA were amplified in the presence of increasing amounts of mammalian DNA showed that the intensity of the 188-bp band decreases as the intensity of the background bands increases (gels not shown).

Since the levels of parasites circulating in the blood of individuals chronically infected with *T. cruzi* may be substantially lower than the eight organisms per 100 μl detected in the previous experiment, we sought to define further the limit of sensitivity of our detection procedure. This effort was made difficult by the fact that blood samples containing very small numbers of parasites cannot be prepared with accuracy. Therefore, we extracted DNA from mouse blood containing a relatively large number of parasites (50,000/ml) and tested the ability of PCR to detect the presence of titrated amounts of parasite DNA (500 to 0.005 parasite equivalents) in the presence of DNA extracted from 100 μl of mouse blood. The mixtures of parasite and mouse DNAs were vortexed extensively after extraction, in an effort to shear the arrays of parasite DNA repetitive elements and facilitate detection. The results obtained by using this experimental approach are presented in Fig. 5A, which shows the products of reactions into which DNA equivalents of 500, 50, 5, 0.5, 0.05, 0.005, and 0 parasites were added (lanes 1 to 7, respectively). In the first three lanes, intense ethidium bromide-stained 188-bp fragments are present, and in lane 4, representing the sample containing the DNA of one-half of a parasite, a lighter band is clearly evident. When these reaction products were blotted onto nitrocellulose and probed with a radiolabeled segment of the 195-bp element located internal to the primers (Fig. 5B), a faint signal above the background level was clearly visible in lane 6, which contained the amplification products of 1/200 of the DNA in a single parasite.

PCR amplification was also performed on DNA extracted from recently collected blood samples from two patients infected with *T. cruzi* (13; P. R. Kerndt, unpublished results), one of whom had a positive xenodiagnosis in 1984 (13). DNA was extracted from 2-ml samples of EDTA-treated blood, and after extensive vortexing, 1/200 of the material, representing that obtained from 10 μl of blood, was added to the reaction mixture. When the amplification products were applied to a nylon membrane and hybridized with the radioactive probe, no signal above background was detected (results not shown).

DISCUSSION

Our choice of the 195-bp repetitive genomic DNA sequence of *T. cruzi* (10, 32) as a target for PCR amplification, rather than the kinetoplast DNA minicircles which have been suggested previously as targets for detection of closely related *Leishmania* spp. (26), was based on several factors. First, there are approximately 1.8 times as many copies of the 195-bp element per organism as there are of the amplifiable minicircle constant regions. This estimate is based on the fact that the minicircles constitute about the same fraction of the total DNA of the organism (9%) as do the 195-bp repeats, but the ~1,450-bp minicircle sequence of *T. cruzi* (7) has only four copies of the 120-bp highly conserved region. Moreover, the 120-bp minicircle region of *T. cruzi* contains some sequence similarity to the replication origins of minicircle DNAs in other trypanosomatids, and this could reduce the specificity of DNA sequence-based detection methods such as PCR amplification. Our demonstration that TCZ1 and TCZ2 do not prime PCR amplification of DNA sequences in several other trypanosomatids suggests that this lack of specificity is not a problem in our assay. In addition, the minicircles of the kinetoplast DNA network are

tightly supercoiled, and cleavage with a restriction enzyme might be necessary to maximize the accessibility of the target sequences to the primers and the *Taq* DNA polymerase. Thus the abundance and specificity of the 195-bp repetitive element make it an ideal target sequence for primer-directed amplification of *T. cruzi* DNA.

In this study, we showed that PCR amplification of the 195-bp *T. cruzi* repetitive element is a highly sensitive method for detecting small numbers of parasites. As few as eight organisms in 100-μl samples of either mouse or human blood were easily detected by ethidium bromide staining of electrophoretically separated reaction products. This level of sensitivity is considerably greater than that obtained in two earlier studies in which 67 and 80 parasites were detected by methods that did not involve amplification of parasite DNA (2, 10). Further definition of the limit of sensitivity of our method is difficult because blood samples containing very few parasites (for example, 1 parasite per 2 ml of blood) cannot be prepared with accuracy. To circumvent this problem, we examined PCR amplification of DNA extracted from mouse blood heavily infected with *T. cruzi* after it was serially diluted and mixed with DNA from uninfected mouse blood. When the radioactive probe was used to detect the amplified 188-bp segment, only about 1/200 of the DNA of a single parasite was necessary for a positive identification. If this high degree of sensitivity was extrapolated to the case of an individual chronically infected with *T. cruzi*, we would expect to be able to detect about 1 parasite in 2 ml of blood. The validity of this extrapolation depends on two assumptions. The first is that the DNA of the parasite can be effectively extracted in the presence of such a relatively large amount of human DNA. Results obtained by other investigators indicate that small amounts of foreign DNA can be efficiently extracted from blood (24). Second, it assumes that the 120,000 copies of the 195-bp element present in a single parasite, which are arranged in tandem arrays for the most part, can be sheared to such an extent that when a fraction of the extracted material representing 10 μl of blood is placed in the reaction mixture, a sufficient number of copies of the target repeat unit are present to allow initiation of amplification. Our findings (Fig. 5) suggest that shearing of the arrays can be accomplished.

Since the PCR-based assay for the detection of *T. cruzi* has a sensitivity many times greater than that described in earlier reports, it was disappointing that no parasites were detected in the two samples of blood from patients chronically infected with *T. cruzi*. This failure is most likely due to the parasitemias being below the limit of detection of the assay. Earlier studies indicate that in individuals with chronic *T. cruzi* infections, parasitemias may be intermittent or so low that samples contain few or no parasites. For example, xenodiagnosis gives positive results in only 50% of patients who clearly have Chagas' disease and may be intermittently positive in individual patients (5). If indeed parasitemias are intermittent, diagnostic methods that detect parasite components rather than the humoral response to the presence of infection may have to be performed repeatedly on individual patients to be of value. PCR amplification is clearly now the method of choice for distinguishing among these possibilities.

Because of the above considerations, DNA detection assays may be more applicable to studies of *T. cruzi* infections in insects than in chronically infected mammalian hosts. As demonstrated here, PCR should be able to detect small numbers of parasites in a field-collected insect by amplifying a fraction of the DNA extracted from the entire abdominal contents, although we have not yet determined the limits of detection in this system. PCR amplification offers two further advantages for field surveys of infected bugs. First, the DNA extractions, amplifications, and analyses of several dozen insects can be conducted simultaneously. In addition, the numbers of parasites in individual insects could be estimated from the intensity of the signal. For example, in the experiments with parasite-mouse DNA mixtures (Fig. 5B), densitometric scanning of the autoradiogram showed that an approximately 100-fold linear range of intensities occurred in the slot blots representing 5, 0.5, and 0.05 parasite equivalents (not shown). In this study, we did not use the intensity of the bands to attempt precise quantitation of an unknown number of parasites in either insects or blood, but such a correlation would certainly be feasible.

Clearly, the next phase in evaluating the usefulness of PCR amplification for *T. cruzi* detection is to apply it in field studies of the prevalence of the parasite in insects and to examine larger numbers of mammalian hosts than are described here.

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