

## Polymerase chain reaction amplification of *Trypanosoma cruzi* kinetoplast minicircle DNA isolated from whole blood lysates: diagnosis of chronic Chagas' disease

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A 6 M guanidine-HCl/0.2 M EDTA solution was used to lyse and store whole blood specimens. DNA stored in guanidine-EDTA-blood (GEB) lysate was found to be undegraded after incubation at 37°C for 1 month, suggesting that this represents an appropriate reagent for transport of blood samples from the field to a laboratory for analysis. *Trypanosoma cruzi* kinetoplast DNA in GEB lysate can be cleaved using the chemical nuclease, 1,10-phenanthroline-copper ion (OP-Cu<sup>2+</sup>). This procedure liberates linearized minicircle molecules from network catenation, distributing them throughout the lysate, and allowing a small aliquot of the original lysate to be analyzed by PCR amplification. This increases the sensitivity of the method dramatically for the detection of small numbers of trypanosomes in a large volume of blood. DNAs isolated from aliquots of *T. cruzi*-positive GEB lysates were polymerase chain reaction (PCR)-amplified with 3 sets of *T. cruzi*-specific kDNA minicircle primers, yielding the 83-bp and 122-bp conserved region fragments and the 330-bp variable region fragments. The PCR products were analyzed by gel electrophoresis and/or hybridization. Results indicate that a single *T. cruzi* cell in 20 ml of blood can be detected by this method. Blood samples from several chronic chagasic patients were tested. Amplification of *T. cruzi* kDNA minicircle sequences was obtained in all cases, even when xenodiagnosis was negative. This PCR-based test should prove useful as a replacement or complement for xenodiagnosis or serology in clinical and epidemiological studies of chronic Chagas' disease.

Key words: *Trypanosoma cruzi*; Chagas' disease; Polymerase chain reaction; Xenodiagnosis; Kinetoplast DNA; Chemical nuclease

### Introduction

Amplification of DNA by the polymerase chain reaction (PCR) has revolutionized DNA-based clinical diagnostic tests. Infectious disease pathogens such as HIV [1], hepatitis B virus [2], and cytomegalovirus [3] are being targeted with this new technology.

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*Abbreviations:* kDNA, kinetoplast DNA; PCR, polymerase chain reaction; OP-Cu<sup>2+</sup>, 1,10 phenanthroline-copper ion; GEB lysate, guanidine/EDTA/blood lysate.

DNA hybridization assays have been developed also for detection of several parasitic protozoa: *Plasmodium* sp. [4,5], *Toxoplasma gondii* [6], *Leishmania* sp. [7] and *Trypanosoma cruzi* [8,9]. There are several advantages to a DNA hybridization diagnostic test: It can be used not only to distinguish parasite species and strains, but the test results are independent of the patient's immunocompetence or previous infection history, thereby allowing an estimation of the current parasitological status of the patient [10].

American trypanosomiasis or Chagas' disease is a major cause of morbidity and mortality in Latin America. The etiological

agent, *Trypanosoma cruzi*, is transmitted by hematophagous triatomid bugs. However, human transmission can occur by alternative routes such as blood transfusion, organ transplantation and congenital transmission. Chagas' disease is characterized by an initial acute phase with high levels of bloodstream parasitemia. The acute phase leads gradually into a life-time chronic phase during which parasites are normally undetectable by microscopic examination of peripheral blood. Diagnosis of chronic infections with *T. cruzi* is unsatisfactory. Current methods include serological tests such as complement fixation, immunofluorescence and enzyme-linked immunosorbent assays. However, serodiagnosis gives rise to a high number of false positive results due to antigenic cross-reactivity with other parasites such as *Leishmania* and *Trypanosoma rangeli* [11]. Xenodiagnosis represents the most specific and sensitive technique currently available for diagnosis of chronic *T. cruzi* infections and is used clinically to confirm the diagnosis of seropositive individuals. However, the lack of standardization of xenodiagnostic procedures makes difficult the comparative analysis of test results. In serologically positive chronic chagasic patients, xenodiagnosis yields positive results in only 17–70% of cases [12], with an overall vector infection rate of 30–100% per test, depending on the vector species used [13–15]. There is also a marked parasite strain dependency for growth of *T. cruzi* in different species of triatomine bugs [16,17], which may lead to flawed clinical and epidemiological studies which rely on xenodiagnosis. Clearly, xenodiagnosis is a flawed diagnostic tool.

We have shown previously that PCR can be used to amplify kinetoplast DNA (kDNA) minicircle sequences which serve as appropriate species- and strain-specific markers for detection and schizodeme classification of *T. cruzi* [8,18]. In this paper we present a method for the storage of human whole blood specimens at room temperature and a procedure for the isolation of kDNA from this blood lysate with subsequent PCR amplification of the kDNA minicircles. The method was used to

detect parasites in blood from a chronic chagasic patient for which xenodiagnosis was negative and should prove useful in clinical and epidemiological studies of Chagas' disease.

## Materials and Methods

**Parasites.** *T. cruzi* epimastigotes (Peru strain) were generously donated by Jerry Manning (University of California at Irvine). Total kDNA from these cells was isolated as described previously [19,20]. Three additional strains, DM28C, Peru and CL, were obtained from Stuart Krassner (University of California at Irvine).

**Infection of laboratory mice.** Approximately 100 000 parasites (40% metacyclic, DM28C) were injected intraperitoneally into mice directly from culture. Mice were bled at 11, 18 and 22 days post-injection. On day 22, motile trypanosomes were observed in venous blood. The strain was passaged every 3 weeks by intraperitoneal injection as maximum parasitemia was observed approximately 22 days post-injection. Blood samples for PCR were obtained from the tail vein and directly diluted 1:1 with 2× GE lysis buffer (see below). Mouse cardiac muscle rinsed with saline was stored in 2× GE lysis buffer.

**Feeding and infection of triatomine insect vectors.** Highly parasitemic mice (approximately 3 *T. cruzi* cells per 1 000× field) were anesthetized. Previously starved (2 weeks) nymphal stages as well as adults of the species, *R. prolixus* and *T. dimidiata*, were allowed to feed upon infected mice. Feeding was repeated at 2-week intervals upon infected mice for a period of 6 weeks. To determine infection with *T. cruzi*, 1 month after initial feeding upon infected mice the bugs were allowed to feed upon uninfected mice. Clear as well as opaque feces were collected after feeding by putting engorged insects into conical tubes containing a few drops of 2× GE lysis buffer, and allowing the bugs to defecate into the buffer.

*GEB lysates.* Human intravenous blood was freshly drawn and collected into tubes containing an equal volume of  $2 \times$  GE lysis buffer (6 M guanidine HCl/0.2 M EDTA, pH 8.0). The resulting GEB (guanidine/EDTA/blood) lysate was stored at 4°C.

*Stability of DNA in GEB lysates.* Plasmid DNA, pGEM 7Z (Promega), was added to 2 tubes of GEB lysates. Each tube was stored at either 37°C or 65°C. Equivalent aliquots from each tube were taken at different time intervals for up to 4 weeks. The aliquots were extracted once with phenol/chloroform (1:1, v/v) and ethanol precipitated. The isolated DNA was electrophoresed in a 1% agarose gel.

*Chemical cleavage of kDNA networks using 1,10-phenanthroline-copper ion.* kDNA resuspended in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) was cleaved in a reaction mixture containing 0.2 mM  $\text{CuSO}_4$ /0.4 mM 1,10-phenanthroline/10 mM  $\text{MgCl}_2$ /5.8 mM 3-mercaptopropionic acid [21]. The 10- $\mu\text{l}$  reaction mixture was incubated at 4°C. The reaction was stopped at different times by addition of 2  $\mu\text{l}$  of 28 mM 2,9-dimethyl-1,10-phenanthroline. The reaction conditions for cleavage of kDNA in GEB lysates were modified as follows: to 1 vol. of GEB lysate containing kDNA, 0.1 vol. of each of the following solutions was added: 1 M  $\text{MgCl}_2$ /200 mM  $\text{CuSO}_4$ /20 mM 1,10-phenanthroline/7.5%  $\text{H}_2\text{O}_2$  (diluted fresh from 30% stock). The reaction was initiated by addition of 0.1 vol. of 58 mM MPA (3-mercaptopropionic acid). Digestion of DNA was allowed to proceed for 30 min at 37°C. The reaction was stopped by addition of 0.1 vol. of 1.5 M DMOP (2,9-dimethyl-1,10-phenanthroline).

*Analysis of 1,10-phenanthroline-copper ion-cleaved kDNA.* GEB lysate containing *T. cruzi* kDNA was digested with OP-Cu<sup>2+</sup> as described above for up to 60 min at 37°C. Aliquots were removed every 10 min and the reaction quenched with DMOP. The aliquots were deproteinized with phenol/chloroform (1:1) and precipitated with ethanol. The

DNA was denatured in glyoxal/DMSO and loaded onto a 1% agarose gel and electrophoresed as described previously [22]. The gel was blotted and the DNA transferred to a Nytran filter (Schuell and Schuster). Total *T. cruzi* kDNA was nick-translated with [<sup>32</sup>P]ATP and used as a hybridization probe. Nick translation and hybridization conditions were as described before [23].

*Isolation of DNA from GEB lysates.* After GEB lysates were digested with OP-Cu<sup>2+</sup> and the reaction quenched as described above, 500- $\mu\text{l}$  aliquots were removed. The aliquots should be processed the same day the GEB lysate is digested with OP-Cu<sup>2+</sup>. Each 500- $\mu\text{l}$  aliquot was extracted once with 100  $\mu\text{l}$  of phenol/chloroform (1:1). The aqueous phase was transferred to an eppendorf tube containing 40  $\mu\text{g}$  of glycogen (2  $\mu\text{l}$  of 20  $\mu\text{g}$   $\mu\text{l}^{-1}$  stock solution) and 50  $\mu\text{l}$  of 3 M NaOAc. Ethanol (1 ml) was added and the DNA precipitated at room temperature by spinning the tubes in a microcentrifuge for 20 min. The pellet was resuspended in 1 ml of water and transferred to a Centricon-100 micro-concentrator (Amicon) containing 1 ml of water. The centricon unit was centrifuged at  $1\,000 \times g$  in a clinical centrifuge for 10 min. The retentate was washed a second time with 2 ml water. After the second 10 min. centrifugation the 100  $\mu\text{l}$  of concentrated retentate was collected as described by the manufacturer. The retentate was used for PCR amplification. The DNA is stable for months when the retentate is stored at -20°C.

*Polymerase chain reaction amplification of minicircle kDNA.* 70  $\mu\text{l}$  of the retentate material was amplified in a 100  $\mu\text{l}$  reaction. The reaction conditions were the following: 10 mM Tris-HCl, pH 8.3/50 mM KCl/5 mM  $\text{MgCl}_2$ /0.1 mg  $\text{ml}^{-1}$  BSA/3 units of *Taq* DNA polymerase (Perkin-Elmer Cetus), and 100 pmol of each primer. The primers used have been described previously [8,18]. The 3 sets of primers anneal to the 4 conserved regions in *T. cruzi* minicircles, yielding the 330-bp minicircle variable region fragments, and the 83- and 122-

bp conserved region fragments, respectively. The cycling profile was as follows: denaturation, annealing and elongation were done at 94°C, 60°C, and 72°C, respectively. Each step was allowed to proceed for 1 min for a total of 30 cycles. A 15- $\mu$ l aliquot from each reaction was analyzed on agarose/Nusieve gels.

**Hybridizations.** PCR-amplified samples analyzed by agarose gel electrophoresis were transferred to Nytran membranes by capillary blotting. Oligo-probe labeling and hybridizations were done as described previously [18].

**Chronic Chagasic patient.** The patient was a 49-year-old Ecuadorean woman who had experienced 3 episodes of sudden cardiac arrest in March 1990 due to ventricular tachycardia. An evaluation in Ecuador revealed normal coronary articles and a positive complement fixation test for *T. cruzi*. In April 1990, she had another episode of ventricular tachycardia and underwent evaluation at UCLA. A *T. cruzi* ELISA test was 1:16 (normal <8).

**Xenodiagnosis.** Uninfected triatomids of several species, from the UCLA laboratory colonies, were allowed to feed upon the patient: 12 *Triatoma dimidiata*, 10 *Triatoma protracta*, and 19 *Rhodnius prolixus* of second, third, fourth and fifth nymphal as well as adult stages were used. Approximately one-third of the bugs fed, and defecation spots were found on the patient. Microscopic examination of bug feces at 30 and 60 days after feeding revealed no *T. cruzi*.

**Polymerase chain reaction-based diagnosis.** Intravenous blood (10 ml) was drawn from the patient on the same day xenodiagnosis was performed, and mixed with an equal volume of 2  $\times$  GE lysis buffer. The GEB lysate was stored at 4°C, and OP-Cu<sup>2+</sup> cleavage, DNA isolation and PCR amplification were performed as described above.

## Results

**Lysis of whole blood with guanidine-EDTA.** Guanidine-HCl is a chaotropic salt commonly used for isolation of nucleic acids from cellular extracts [24,25]. The ability of this reagent to disrupt cells and inhibit nucleases makes it ideal for preservation of nucleic acids in biological fluids. To inhibit coagulation of blood, 0.2 M EDTA was added to the 6 M guanidine lysis solution (2  $\times$  GE = 6 M guanidine HCl/0.2 M EDTA, pH 8.0). Our standard lysis procedure was to mix an equal volume of whole blood and 2  $\times$  GE at room temperature.

**Stability of closed circular DNA in guanidine lysate.** To assay the stability of DNA in GEB lysate, closed circular plasmid DNA was added to the lysate and the percentage of nicked or linearized plasmid molecules assayed after

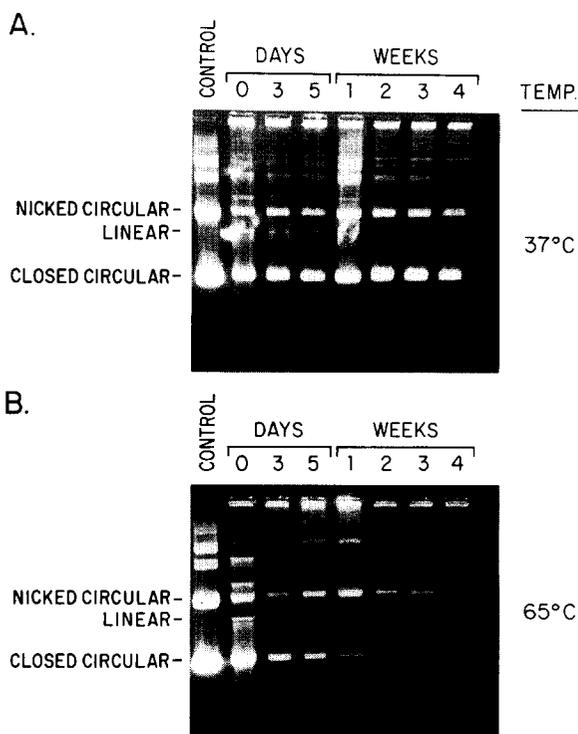


Fig. 1. Stability of DNA in GEB lysate. Plasmid pGEM 7Z DNA was incubated in GEB lysate at 2 different temperatures. (A) 37°C incubation. (B) 65°C incubation. Control lane, plasmid DNA in 10 mM Tris-HCl pH 8, 1 mM EDTA (TE). 1% agarose gel.

different periods of incubation at 37°C or 65°C. Fig. 1 shows that the DNA remains intact at 37°C for at least a month, with no apparent nicking or degradation. At 65°C, the DNA is nicked after a 2 week incubation, as indicated by the disappearance of the closed circular DNA band and the increase in the nicked circular band. Even at 65°C, at least 50% of the DNA remained in the nicked circular or linear form after 1 week incubation. These results clearly indicate that the GE reagent is a suitable medium for the lysis of whole blood and the preservation of DNA at room temperature for some time. At 4°C or -20°C, DNA is stable in GEB lysate for at least a year (data not shown).

*1,10-Phenanthroline-copper ion digestion of kDNA networks releases linearized minicircle molecules.* The catenation of  $10^4$  minicircles into the single kDNA network present in the *T. cruzi* cell greatly affects the sensitivity of amplification analysis by decreasing the DNA target number per unit volume of blood lysate. A chemical cleavage method, using 1,10-phenanthroline- $\text{Cu}^{2+}$  ion (OP- $\text{Cu}^{2+}$ ), was employed to produce a single double-strand cleavage per minicircle molecule, thereby releasing the individual minicircles as linear molecules and distributing the amplification target molecules throughout the lysate. This allows a small aliquot of the original lysate to be processed which would still contain sufficient minicircle molecules for amplification. OP- $\text{Cu}^{2+}$  introduces random single-strand nicks into duplex DNA in the presence of peroxide [26]. Since, on the average, one double strand cleavage occurs after approximately 10 random single strand breaks are introduced into a DNA molecule, this reagent can be used to digest network kDNA to linearized minicircles. The extent of cleavage of purified *T. cruzi* kDNA in buffer was monitored by agarose gel electrophoresis as a function of digestion time. Undigested network kDNA remains in the well since, due to its large size, it cannot enter the gel. After 1 min digestion, very little network kDNA can be detected in the well and the released

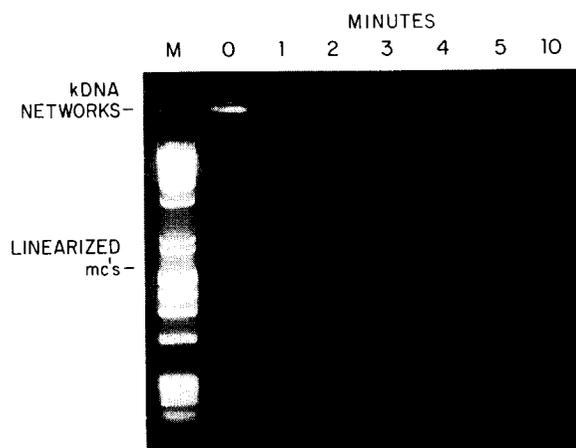


Fig. 2. OP- $\text{Cu}^{2+}$  cleavage of *T. cruzi* kDNA networks. Isolated kDNA was resuspended in TE and digested with OP- $\text{Cu}^{2+}$  for different periods of time. Notice the disappearance of the kDNA from the well and the appearance of the 1.4-kb linear minicircle band as a function of digestion time. After 10 min, the DNA is completely degraded. M lane,  $\lambda$  DNA (*Hind*III fragments)/ $\phi$  X 174 (*Hae*III fragments). 1% agarose gel.

linearized minicircle DNA migrates as a 1.4-kb band (Fig. 2). Continued digestion leads to the appearance of a lower-molecular-weight smear resulting from multiple cleavages per minicircle molecule, but the reaction can be terminated at any point by the addition of DMOP, a  $\text{Cu}^{2+}$ -chelating agent. The OP- $\text{Cu}^{2+}$  reagent is also active in cleaving minicircle DNA in the GEB lysate, with minor modifications as described in Methods. The modifications include increasing the  $\text{Cu}^{2+}$  concentration and the  $\text{H}_2\text{O}_2$  concentration, and increasing the time and temperature of incubation.

*Extent of single-strand nicks in linearized minicircle molecules.* If the duplex minicircle fragments released from the network contain excessive single-strand nicks, the DNA will not be an adequate substrate for PCR amplification. Upon denaturation, the size of the single-stranded fragment has to be at least equal to the distance between the 2 PCR primers in order to obtain successful amplification. kDNA was cleaved with OP- $\text{Cu}^{2+}$  in GEB lysate for increasing periods of time and the cleaved DNA was electrophoresed in a denaturing glyoxal gel to determine the size

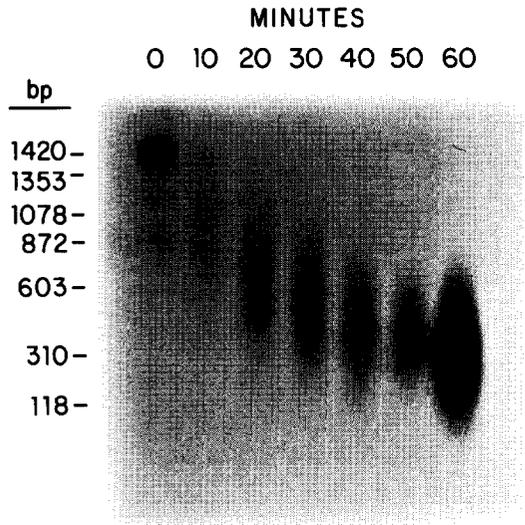


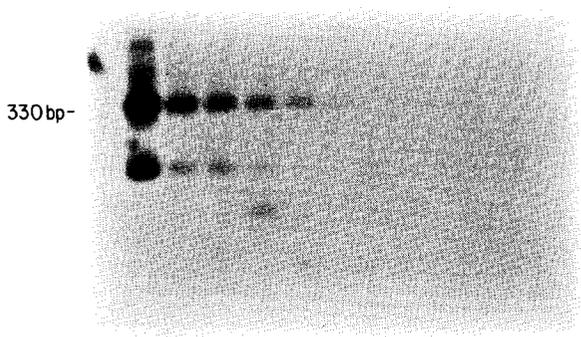
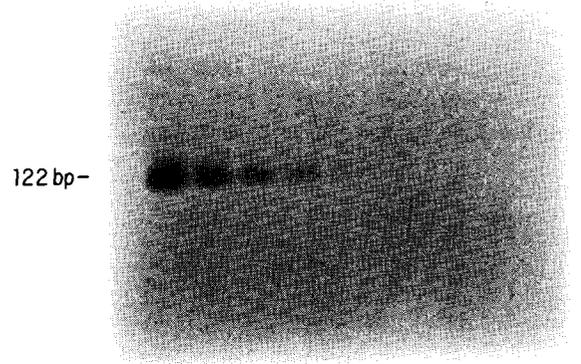
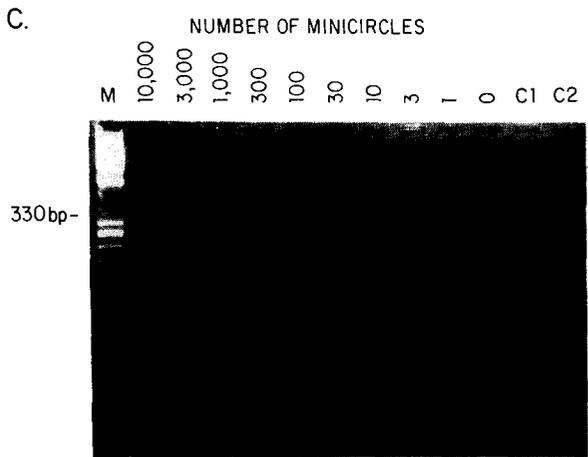
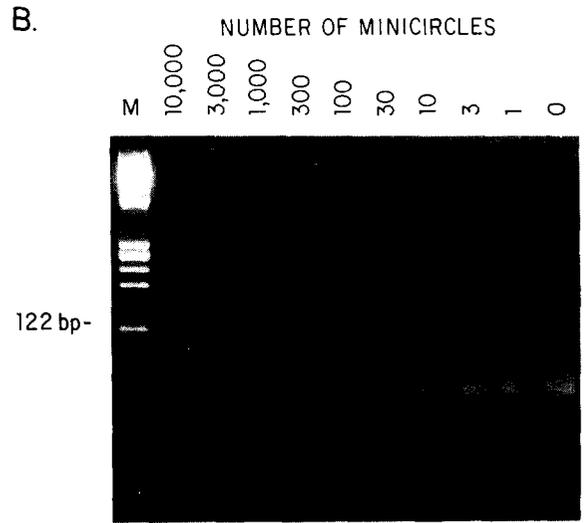
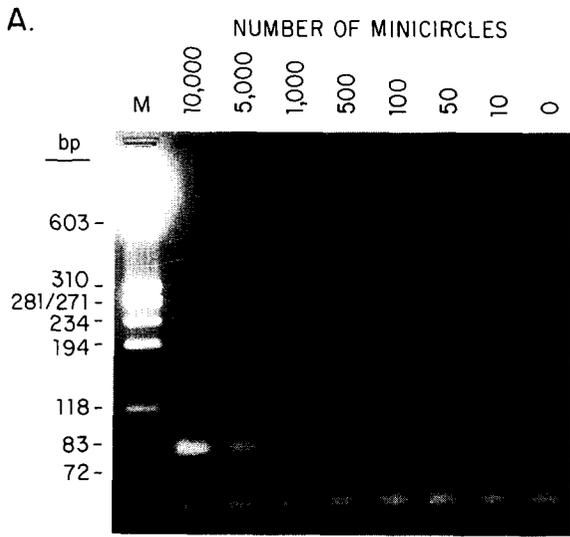
Fig. 3. Frequency of single strand nicks in OP-Cu<sup>2+</sup>-linearized minicircle DNA. *T. cruzi* kDNA networks in GEB lysate were cleaved with OP-Cu<sup>2+</sup> for increasing periods of time. After cleavage the DNA was electrophoresed in a denaturing glyoxal gel (1% agarose), blotted onto a nylon membrane and probed with <sup>32</sup>P-labeled kDNA. At time 0, a few decatenated minicircles undergoing replication can be seen as a 1.4 kb band. After OP-Cu<sup>2+</sup> cleavage, the minicircles are released from the kDNA networks as double stranded linearized molecules. By denaturing the linearized minicircles, it can be seen that the minicircles are increasingly being nicked as a function of digestion time. After 30 min, 90% of the minicircle fragments are larger than 310 bp. After 60 min 50% of the fragments are larger than 310 bp, and 80% of the fragments are larger than 118 bp.

distribution of single-strand fragments. The gel was blotted and hybridized with <sup>32</sup>P-labeled *T. cruzi* kDNA. Fig. 3 shows that with increasing incubation time there is a decrease in the size of the single-stranded fragments. However, even after a 60-min incubation, 80% of the denatured minicircle fragments are longer than 118 bp, and approximately half of the

fragments are above 310 bp. We selected an incubation time of 30 min for routine OP-Cu<sup>2+</sup> digestion of blood lysates, at which time approximately 90% of the single-stranded fragments are longer than 310 bp, and consequently would be appropriate amplification target molecules for the 3 sets of PCR primers which yield products of 83 bp, 122 bp, and 330 bp, respectively [8]. A control experiment showed that kDNA digested with OP-Cu<sup>2+</sup>, in GEB lysate under standard conditions for 30 min, was a suitable template for PCR amplification (data not shown).

*Sensitivity titration of 1,10-phenanthroline-copper ion-cleaved minicircle DNA.* To determine the sensitivity of the PCR-minicircle DNA assay, the equivalent of 20 *T. cruzi* kDNA networks was added to 10 ml GEB lysate. The network DNA was cleaved with OP-Cu<sup>2+</sup> as described in Materials and Methods. After cleavage, the GEB lysate was diluted with kDNA-free GEB lysate to give DNA concentrations of 1–10 000 minicircles per 500  $\mu$ l of GEB lysate. Total DNA was isolated from these aliquots as described in Methods and subjected to PCR amplification of the 3 minicircle-specific fragments. As shown in Fig. 4, at least 100 minicircles in 500  $\mu$ l of GEB lysate can be detected, either by ethidium bromide staining (83 bp PCR product) or by hybridization with <sup>32</sup>P-labeled oligonucleotide probes (122-bp and 330-bp PCR products). Therefore, this procedure can detect the equivalent of 1% of the minicircle content of a single *T. cruzi* cell in 500  $\mu$ l of OP-Cu<sup>2+</sup>-digested GEB lysate. These results suggest that this procedure could be used for

Fig. 4. Sensitivity titration of OP-Cu<sup>2+</sup>-cleaved kDNA. *T. cruzi* kDNA was OP-Cu<sup>2+</sup>-digested in GEB lysate and diluted with kDNA-free GEB lysate. 500  $\mu$ l aliquots of GEB lysate containing decreasing numbers of minicircle molecules (calculated from the DNA concentration) were processed as described in Methods. The isolated DNA was PCR amplified and 1/6 of each PCR reaction was loaded onto a gel. M lane,  $\phi$  X 174 (*Hae*III fragments). (A) Amplification of 83-bp fragments. 35 cycles. 2% agarose/3% Nusieve gel. The rapidly migrating band present in all lanes represents PCR primers or primer dimers. (B) Amplification of 122-bp fragments. 30 cycles. Top panel, stained 1% agarose/3% Nusieve. The low molecular band present in all lanes represents PCR primers. Bottom panel, hybridization of blot with <sup>32</sup>P-labeled S34A oligonucleotide internal probe [8]. (C) Amplification of 330-bp variable region fragments. 30 cycles. Top panel, stained 1% agarose/3% Nusieve gel. Bottom panel, hybridization of blot with <sup>32</sup>P-labeled S67 oligonucleotide internal probe [8,18]. The identity of the rapidly migrating labeled band is not known. C1 and C2 lanes, 500  $\mu$ l of undigested (no OP-Cu<sup>2+</sup> cleavage) GEB lysate sample containing 10 kDNA networks per 30 ml, processed for PCR amplification as described for all other samples.



the detection of a single *T. cruzi* cell in 20 ml of blood. This level of sensitivity for detection of *T. cruzi* in blood is at least equivalent, if not better, than that obtained with xenodiagnosis.

**Diagnosis of chronic Chagas' disease.** A 49-year-old female patient from Ecuador showing cardiac disturbances tested positive for *T. cruzi* in two different serological tests – complement fixation and ELISA. Xenodiagnosis (performed by R. Barr, UCLA School of Public Health) was negative. A 10-ml sample of venous blood from the patient was obtained and stored as a GEB lysate. OP-Cu<sup>2+</sup> cleavage of the lysate was performed and DNA was isolated from two 500- $\mu$ l aliquots and PCR-amplified as described in Materials and Methods. Fig. 5 shows the specific amplification of *T. cruzi* minicircle sequences from patient blood with 2 different sets of PCR primers. Blood samples from 4 additional serology-positive chronic chagasic patients (3 patients were xenodiagnosis-negative and one

xenodiagnosis-positive) have also tested positive for *T. cruzi* parasites by PCR amplification of minicircle DNA using our standard procedure (H. Avila, C. Morel, W. Degraeve and L. Simpson, unpublished results). These results indicate that the method should prove useful in diagnosis of chronic Chagas' disease.

**Polymerase chain reaction analysis of triatomine feces and mouse biopsy tissue.** The GEB lysate amplification method was extended to the insect vector and to biopsy material from infected mice. The abdominal contents of 2 *T. dimidiata* and 2 *R. prolixus* bugs were collected and stored in GE buffer. The samples were processed as described in Materials and Methods. Fig. 6 shows the specific amplification of kDNA minicircle sequences from the insect feces. Animal biopsy material can also be dissolved and stored in GE. Heart tissue obtained from infected and uninfected mice was dissolved by incubation in GE at 37°C for 2 days. The dissolved tissue was processed as

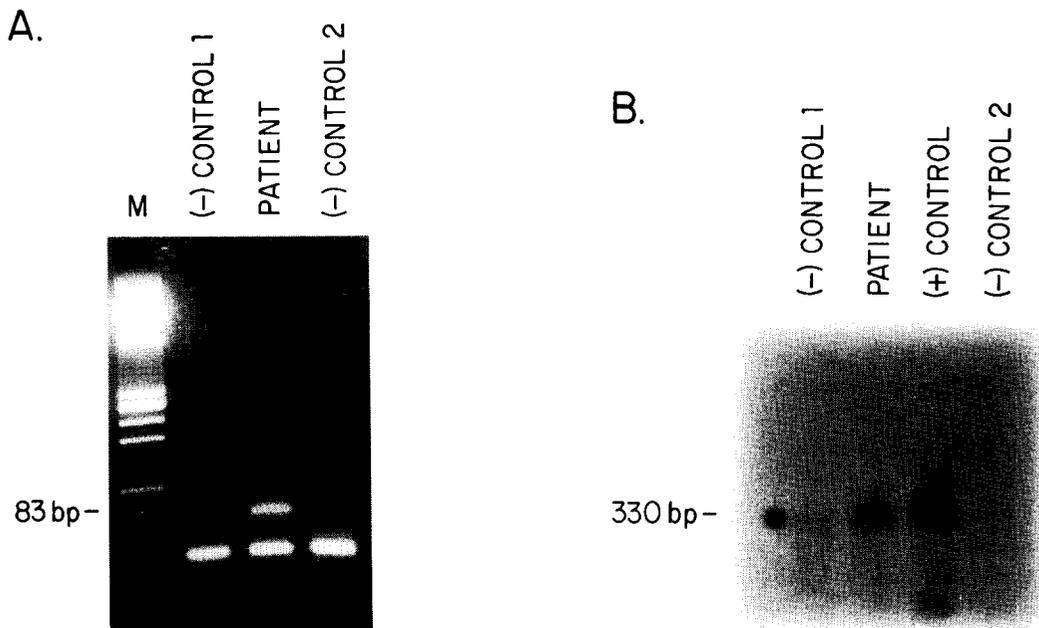


Fig. 5. Blood sample of chronic chagasic patient analyzed for the presence of *T. cruzi* minicircle sequences. Sample was processed as described in Methods. (A) Amplification of 83 bp fragment. Negative control 1 lane, GEB sample from non-chagasic donor. Negative control 2 lane, no kDNA in PCR reaction. M lane,  $\phi$  X 174 (*Hae*III fragment). 1% agarose/3% Nusieve gel. (B) Amplification of 330-bp minicircle variable region fragments. 2% agarose gel, blotted and hybridized with <sup>32</sup>P-labeled S67 oligonucleotide internal probe [8,18]. Positive control, 100 fg gel-isolated OP-Cu<sup>2+</sup>-cleaved kDNA. Negative controls, same as in (A).

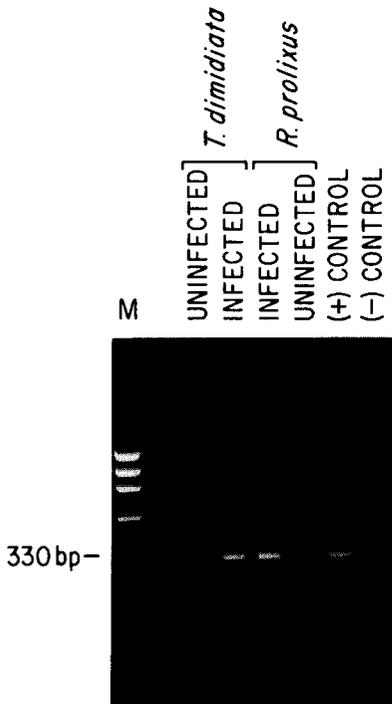


Fig. 6. Polymerase chain reaction amplification of *T. cruzi* kDNA isolated from triatomine bug feces. 330-bp minicircle variable region fragments were amplified. 15  $\mu$ l of PCR reaction (100  $\mu$ l total volume) was loaded in a 2% agarose gel. M lane,  $\phi$  X 174 (*Hae*III fragments). Positive and negative controls are 1  $\mu$ g kDNA and no kDNA, respectively, in PCR reaction.

described. Specific PCR amplification of minicircle sequences was observed from the heart tissue lysates of the infected mouse but not from the uninfected controls (data not shown).

## Discussion

We have shown previously by an analysis of 56 strains of *T. cruzi* from throughout Latin America that the minicircle-specific PCR primers and hybridization probes are species-specific and strain-independent [8,18]. Furthermore, amplification of the minicircle variable region can serve as a marker to distinguish schizodeme groups [18]. We propose to amplify the conserved region 122-bp minicircle fragments and the variable region 330-bp

fragments. Detection of amplified DNA can be accomplished either by gel electrophoresis or by hybridization with specific internal oligomer probes.

In this paper we introduce a procedure for the storage and processing of blood specimens, which in combination with a PCR amplification step will allow the screening of chronic chagasic patients for low numbers of circulating parasites. The 2 $\times$  GE lysis and storage reagent should prove valuable for the recovery and transport of blood samples from the field in endemic countries. Another lysis buffer with similar properties has been reported, consisting of a mixture of Na-Sarkosyl, EDTA and cesium trifluoroacetate [4]. The main advantage of the GE reagent is low cost and compatibility with the OP-Cu<sup>2+</sup> chemical nuclease reagent. We showed that DNA survives in GEB at 37°C for at least 1 month and at 65°C for 1 week without degradation, thereby allowing sufficient time for the samples from endemic regions to arrive at laboratories with refrigeration facilities.

Another novel feature of our assay is the cleavage of the kDNA network into free linearized minicircle molecules by means of a chemical nuclease, OP-Cu<sup>2+</sup>, which is active in the GEB lysis medium. This step effectively increases dramatically the number of target DNA molecules per unit volume of lysate. For example, kDNA from a single trypanosome in 20 ml of GEB lysate would probably be lost during deproteinization; whereas after cleavage of the 10<sup>4</sup> catenated minicircles in the network, a 0.5-ml aliquot would contain 250 linear minicircles. The deproteinization, precipitation and Centricon filtration steps that follow the OP-Cu<sup>2+</sup>-cleavage step are necessary for the removal of inhibitors of PCR [27] present in the blood lysate. A sensitivity titration experiment has shown that we can detect a minimum of approximately 100 minicircle molecules from a 0.5 ml GEB lysate aliquot, after OP-Cu<sup>2+</sup> cleavage. Hence, detection of a single trypanosome in 20 ml of blood is clearly possible. Although the number of circulating parasites in chronic patients is unknown, an estimate may be obtained from the amount of

blood used in the xenodiagnosis protocol. Approximately 20–40 triatomid bugs are used per xenodiagnosis, which take up on average 0.1–0.5 ml of blood per bug, depending on the bug species and instar stage used [15,17,28], for a total volume of 4–20 ml of blood per test.

In an initial test of the method, the GEB amplification procedure was used with blood from a chronic chagasic patient who had 2 positive serology tests and a negative xenodiagnosis and also from 4 other chronic chagasic patients, 3 of which were xenodiagnosis-negative. We were able to isolate and amplify *T. cruzi* minicircle sequences from all of these samples. This is the first report of a successful DNA-based assay for the diagnosis of chronic chagasic patients [9,29]. Clearly, more chronic patients must be examined in a double blind fashion to ascertain the level of false positives and the sensitivity of this assay, but it appears from these initial results that this PCR-based assay is more sensitive than xenodiagnosis and may eventually replace it as the standard test for chronic Chagas' disease.

Another possible use of this assay is for epidemiological studies of *T. cruzi*-infected insects and animals. We have shown that the GE lysis method can be used to recover parasite DNA from infected insects and from heart tissue of infected mice for minicircle amplification. In general, a wide variety of biological specimens could be stored in the GE lysis buffer for eventual DNA isolation and PCR-based assays.

Finally, the GEB procedure may prove valuable in the development of a multiplex PCR assay for a variety of blood-borne parasitic and viral agents. The OP-Cu<sup>2+</sup> cleavage step which is required for analysis of trypanosome DNA should not affect amplification of DNA fragments less than one kb in size and may even prove advantageous for amplification of repetitive target sequences. This method could eventually be used as a rapid, low cost, sensitive screening assay for the presence of various etiological agents in blood from patients or from blood banks.

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## References

- 1 Perrin, L.H., Yerly, S., Adami, N., Bachmann, P., Btler-Brunner, E., Burckhardt, J. and Kawashima, E. (1990) Human immunodeficiency virus DNA amplification and serology in blood donors. *Blood* 76, 641–645.
- 2 Keller, G.H., Huang, D.-P., Shih, J.W.-K. and Manak, M.M. (1990) Detection of hepatitis B virus DNA in serum by polymerase chain reaction amplification and microtiter sandwich hybridization. *J. Clin. Microbiol.* 28, 1411–1416.
- 3 Shibata, D., Martin, W.J., Appleman, M.D., Causey, D.M., Leedom, J.M. and Arnhem, N. (1988) Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus. *J. Infect. Dis.* 158, 1185–1192.
- 4 Zolg, J.W., Scott, E. and Wendlinger, M. (1988) High salt lysates: A simple method to store blood samples without refrigeration for subsequent use with DNA probes. *Am. J. Trop. Med. Hyg.* 39, 33–40.
- 5 Wirth, D.F., Rogers, W.O., Barker, R., Dourado, H., Suesebang, L. and Albuquerque, B. (1989) Leishmaniasis and malaria: DNA probes for diagnosis and epidemiologic analysis. *Ann. NY Acad. Sci.* 569, 183–192.
- 6 Burg, J.L., Grover, C.M., Pouletty, P. and Boothroyd, J.C. (1989) Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.* 27, 1787–1792.
- 7 Rodgers, M.R., Popper, S.J. and Wirth, D.F. (1990) Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Exp. Parasitol.* 71, 267–275.
- 8 Sturm, N.R., Degrove, W., Morel, C. and Simpson, L. (1989) Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' Disease. *Mol. Biochem. Parasitol.* 33, 205–214.
- 9 Moser, D.R., Kirchhoff, L. and Donelson, J.E. (1989) Detection of *Trypanosoma cruzi* by DNA amplification

- using the polymerase chain reaction. *J. Clin. Microbiol.* 27, 1477-1482.
- 10 Barker, R.H., Jr. (1990) DNA probe diagnosis of parasitic infections. *Exp. Parasitol.* 70, 494-499.
  - 11 Manson-Bahr, P.E.C. and Bell, D.R. (1987) *Manson's Tropical Diseases*. Bailliere Tindall, London.
  - 12 Segura, E.L. (1987) Xenodiagnosis. In: *Chagas' disease vectors*, Volume II. Anatomic and Physiological Aspects (Brenner, R.R. and Stoka, A.M., eds.), pp. 41-45. CRC Press, Boca Raton.
  - 13 Perlowagora-Szumlewicz, A. and Muller, C.A. (1987) Studies in search of a suitable experimental insect model for xenodiagnosis of hosts with Chagas' disease. 2. Attempts to upgrade the reliability and the efficacy of xenodiagnosis in chronic Chagas' disease. *Mem. Inst. Oswaldo Cruz* 82, 259-272.
  - 14 Perlowagora-Szumlewicz, A., Muller, C.A. and de Carvalho Moreira, C.J. (1988) Studies in search of a suitable experimental insect model for xenodiagnosis of hosts with Chagas' disease, 3. On the interaction of vector species and parasite strain in the reaction of bugs to infection by *Trypanosoma cruzi*. *Rev. Saude. Publ.* 22, 390-400.
  - 15 Cuba, C.C., Alvarenga, N.J., Barreto, A.C., Marsden, P.D., Macedo, V. and Gama, M.P. (1979) *Dipetalogaster maximus* (Hemiptera, Triatominae) for xenodiagnosis of patients with serologically detectable *Trypanosoma cruzi* infection. *Trans. R. Soc. Trop. Med. Hyg.* 73, 524-527.
  - 16 Miles, M.A., Patterson, J.W., Marsden, P.D. and Minter, D.M. (1975) A comparison of *Rhodnius prolixus*, *Triatoma infestans* and *Panstrongylus megistus* in the xenodiagnosis of a chronic *Trypanosoma (Schizotrypanum) cruzi* infection in a rhesus monkey (*Macaca mullata*). *Trans. R. Soc. Trop. Med. Hyg.* 69, 377-382.
  - 17 Minter, D.M., Minter-Goedbloed, E. and Marshall, T.F.C. (1978) Comparative xenodiagnosis with three triatomine species of different hosts with natural and experimental chronic infections with *Trypanosoma (Schizotrypanum) cruzi*. *Trans. R. Soc. Trop. Med. Hyg.* 72, 84-91.
  - 18 Avila, H., Goncalves, A.M., Nehme, N.S., Morel, C.M. and Simpson, L. (1990) Schizodeme analysis of *Trypanosoma cruzi* stocks from South and Central America by analysis of PCR-amplified minicircle variable region sequences. *Mol. Biochem. Parasitol.* 42, 175-188.
  - 19 Morel, C., Chiari, E., Camargo, E., Mattei, D., Romanha, A. and Simpson, L. (1980) Strains and clones of *Trypanosoma cruzi* can be characterized by restriction endonuclease fingerprinting of kinetoplast DNA minicircles. *Proc. Natl. Acad. Sci. USA* 77, 6810-6814.
  - 20 Gomez-Eichelmann, M.C., Holz, G., Jr., Beach, D., Simpson, A.M. and Simpson, L. (1988) Comparison of several lizard *Leishmania* species and strains in terms of kinetoplast minicircle and maxicircle DNA sequences, nuclear chromosomes, and membrane lipids. *Mol. Biochem. Parasitol.* 27, 143-158.
  - 21 Kuwabara, M.D. and Sigman, D.S. (1987) Footprinting DNA-protein complexes in situ following gel retardation assays using 1,10-phenanthroline-copper ion: *Escherichia coli* RNA polymerase promoter complexes. *Biochemistry* 26, 7234-7238.
  - 22 McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* 74, 4835-4838.
  - 23 Simpson, A., Neckelmann, N., de la Cruz, V., Muhich, M. and Simpson, L. (1985) Mapping and 5' end determination of kinetoplast maxicircle gene transcripts from *Leishmania tarentolae*. *Nucleic Acids Res.* 13, 5977-5993.
  - 24 Bowtell, D.D.L. (1987) Rapid isolation of eukaryotic DNA. *Anal. Biochem.* 162, 463-465.
  - 25 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
  - 26 Sigman, D.S. and Chen, C.B. (1990) Chemical nucleases: new reagents in molecular biology. *Annu. Rev. Biochem.* 59, 207-236.
  - 27 de Franchis, R., Cross, N.C.P., Foulkes, N.S. and Cox, T.M. (1988) A potent inhibitor of *Taq* polymerase copurifies with human genomic DNA. *Nucleic Acids Res.* 16, 10355.
  - 28 Minter-Goedbloed, E., Minter, D.M. and Marshall, T.F.C. (1978) Quantitative comparison between xenodiagnosis and haemoculture in the detection of *Trypanosoma (Schizotrypanum) cruzi* in experimental and natural chronic infections. *Trans. R. Soc. Trop. Med. Hyg.* 72, 217-225.
  - 29 Ashall, F., Yip-Chuck, D.A.M., Luquetti, A.A. and Miles, M.A. (1988) Radiolabeled total parasite DNA probe specifically detects *Trypanosoma cruzi* in mammalian blood. *J. Clin. Microbiol.* 26, 576-578.