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Research Note

# A SIMPLE PROTOCOL FOR THE PHYSICAL CLEAVAGE OF *TRYPANOSOMA CRUZI* KINETOPLAST DNA PRESENT IN BLOOD SAMPLES AND ITS USE IN POLYMERASE CHAIN REACTION (PCR)-BASED DIAGNOSIS OF CHRONIC CHAGAS DISEASE

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The direct parasitological diagnosis of chronic Chagas' disease usually relies on xenodiagnosis, or the biological amplification in insect vectors of living *Trypanosoma cruzi* circulating in peripheral blood. The detection of *T. cruzi* by the biochemical amplification of parasite DNA sequences by the polymerase chain reaction (PCR) has been demonstrated and proposed as a potentially useful alternative approach to diagnostic and epidemiological studies (N. Sturm et al., 1989, *Mol. Biochem. Parasitol., 33:* 205-214; H. Avila et al., 1990, *Mol. Biochem. Parasitol., 42:* 175-188; C. Diaz et al., 1992, *Am. J. Trop. Med. Hyg., 46:* 616-623; V. Russomondo et al., 1992, *J. Clin. Microbiol., 30:* 2864-2868; H. Avila et al., in preparation).

When kinetoplast DNA (kDNA) is used as the target for PCR amplification, as proposed by N. Sturm et al. (*loc. cit.*), the kinetoplast networks must be cleaved in order to liberate the catenated minicircles and distribute the target sequences homogeneously in the blood sample. This has been successfully acomplished by the use of the chemical nuclease copper/phenanthroline which dramatically improved the sensitivity of PCR-based detection of *T. cruzi* (H. Avila et al., 1991, *Mol. Biochem. Parasitol., 48:* 211-222), allowing the efficient diagnosis of chronic Chagas disease in human patients (H. Avila et al., in preparation).

The chemical cleavage protocol, however, introduces the need for extra manipulation of the blood samples and therefore increases the possibility of cross contamination and of false positive in PCR-based diagnostic protocols. We have found that physical cleavage of kDNA networks can be efficiently accomplished by simply boiling the guanidine-EDTA-blood lysates (GEB lysates, see H. Avila et al., 1991, *loc. cit.*). This finding allowed us to develop a simple, rapid, reliable and less expensive protocol for the PCR-based detection of *T. cruzi* useful in diagnostic, clinical and epidemiological studies.

Figure 1A shows the electrophoretic analysis of kDNA networks which have been boiled for different periods in TE buffer (10 mM Tris-HCl pH 7.0, 1 Mm EDTA). Native networks are formed mainly by catenated minicircles which cannot enter the gel and remain at the origin. Heating for 5 min at 100 °C causes the linearization of the majority of the minicircles, which are liberated from the networks and migrate with a predicted molecular weight of 1.4 kb. Longer boiling times induces further fragmentation of the minicircle sequences which form a smear in the region of lower MW.

The high-temperature fragmentation of kDNA networks in GEB lysates was analyzed by boiling reconstituted blood samples containing a constant amount of kDNA for different periods of time. The heated samples were phenol-extracted and the DNA electrophoresed on a denaturing gel. The migration of the kDNA was thereafter observed by hybridisation with a cloned minicircle probe after transfer of the gel to a nylon membrane. As shown in figure 1B, boiling times of 5-30 minutes liberate large amounts of linearized minicircles. Longer boiling periods induce further degradation of the molecules and again a smear of lower MW can be observed.

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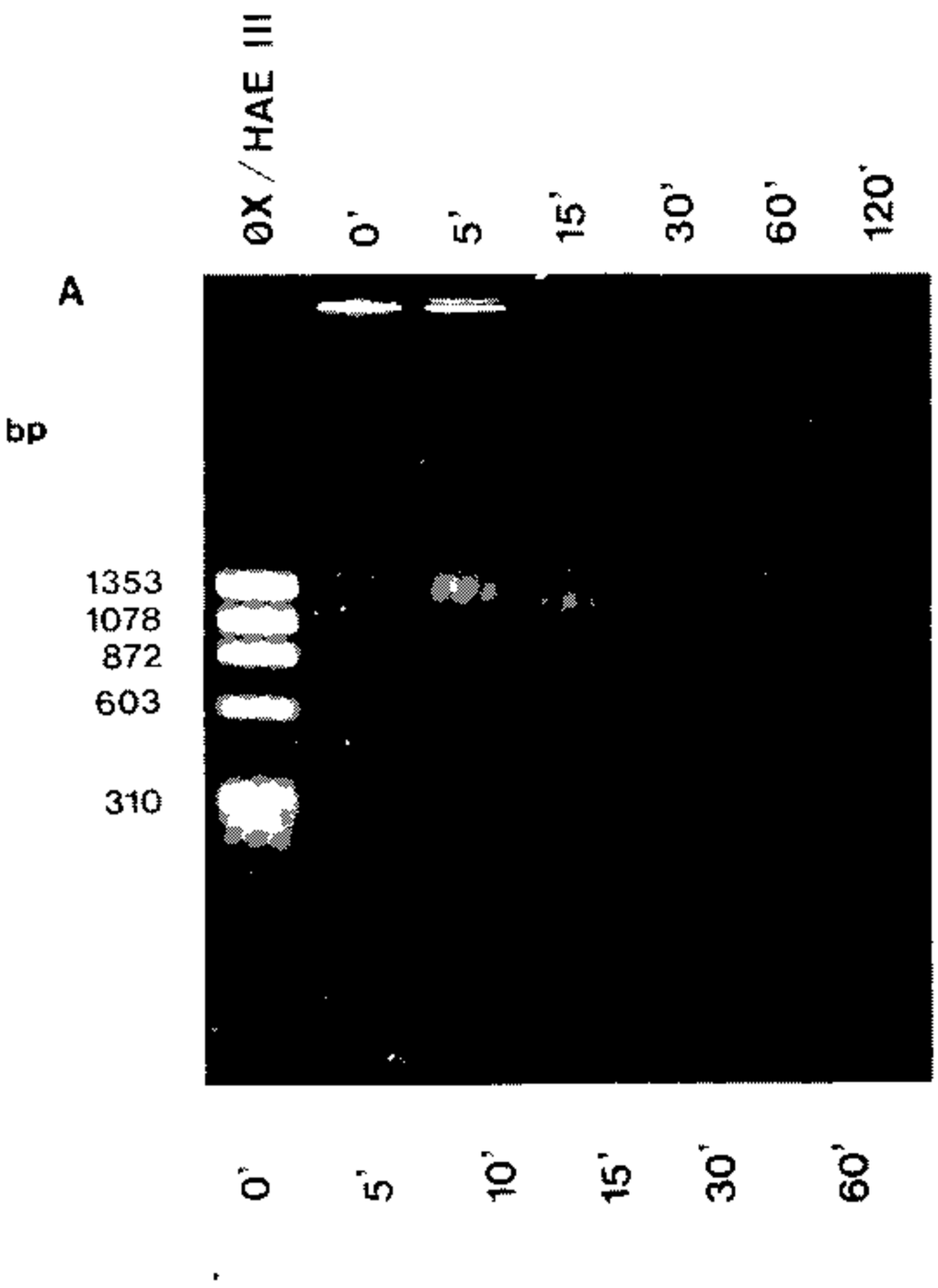



Fig. 1A: effect of the heating of kDNA networks in TE buffer. One µg of kDNA from *T. cruzi* strain Y was boiled for the indicated times and electrophoresed on 1.5% agarose gel. B: degradation of kDNA boiled in blood lysates. One µg of kDNA from *T. cruzi* strain Y was included in one ml samples of previously uncontaminated GEB lysate. After boiling for the indicated times, the purified DNA samples were electrophoresed on a 1.2% agarose gel containing formaldehyde and transfered to a nylon membrane. Hybridisation was made with a $P^{32}$ labeled cloned minicicle (W. Degrave et al., 1988, *Mol. Biochem. Parasitol., 27:* 63-70).

We further made reconstruction experiments to determine the sensitivity of the PCR amplification of minicircle sequences using the boiling method. Five kDNA networks (corresponding to about $10^6$ minicircles) were boiled in one milliliter of blood from a healthy donor for 20 min. Successive dilutions of this sample were then realized in previously boiled blood lysate up to a concentration of 10 minicircle molecules per ml. One aliquot (100 µl) of each dilution was submitted to phenol-chloroform extraction, ethanol precipitation and resuspension in water. These samples were then amplified by PCR. As shown in Fig. 2, the expected product of 300 bp appears when at least $10^3$ minicircles are present per ml of GEB lysate. As a single *T. cruzi* cell contains around $2.10^4$ individual minicircles, we conclude that this procedure allows the detection of one parasite in at least 20 ml of GEB lysate. This sensitivity seems appropriate for an accurate diagnostic of chronic forms of Chagas' disease where the level of circulating trypanosomes is usually low.

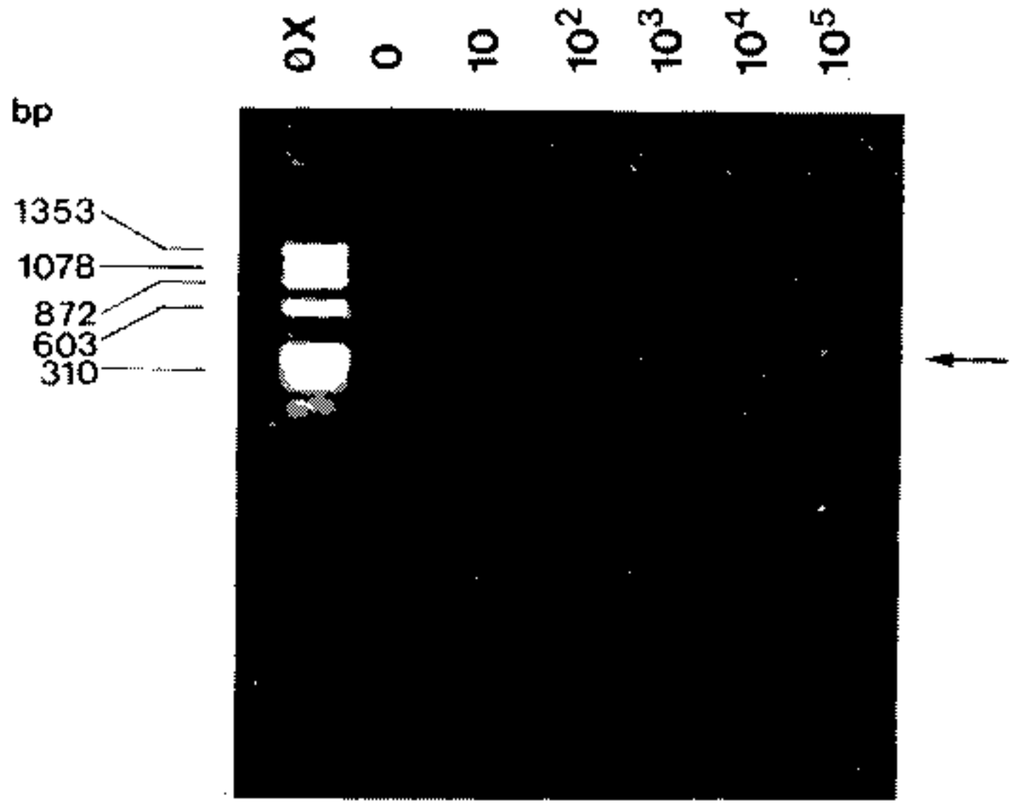


Fig. 2: PCR amplification of diluted minicircles. One hundred µl of GEB lysate containing successive concentrations of minicircle molecules from a previously boiled sample (10 to $10^5$ molecules per ml) were PCR amplified for 25 cycles using primers homologous to constant regions sequences (H. Avila et al., 1991, *loc. cit.*). The arrow points to the expected product of 330 bp. The "O" concentration represents the same GEB lysate used to make dilutions without addition of kDNA.