EXPERIMENTAL PALEOPARASITOLOGY: IDENTIFICATION OF *TRYPANOSOMA CRUZI* DNA IN DESICCATED MOUSE TISSUE

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Summary

The polymerase chain reaction (PCR) has been used for clinical diagnosis of infectious disease and to research ancient animal and microbiological DNA from a wide range of tissues. PCR was used to study the possibility of *Trypanosoma cruzi* kinetoplast DNA (kDNA) extraction from experimentally desiccated mouse tissue (heart, skeletal muscle, liver, spleen, and pancreas). The results obtained suggest the application of this technique to *T. cruzi* detection in archaeological material.

Introduction

Paleoparasitology has developed through the study of parasites in archaeological material. The main source for that research is the analysis of coprolites or latrine soils, and intestinal helminths are the common findings in paleopathological research. Protozoal cysts in coprolites do not seem to be as well preserved as helminth larvae and eggs (Reinhard et al 1988). Lice (*Pediculus humanis*) and fleas (*Pulex irritans*), were recovered from Viking Age house deposits in Greenland (Sadler 1990). *Trypanosoma cruzi* was described in a Peruvian mummy by immuno-histochemistry and ultrastructural study (Fornaciari et al 1992). *Schistosoma* (Miller et al 1992) and *Plasmodium falciparum* infections (Miller et al 1994) were diagnosed in mummies also by using an immunohistochemistry test (Miller et al 1994). PCR was used to identify *Mycobacterium tuberculosis* in ancient skeletons (Spigelman & Lemma 1993) and in mummified lung tissue (Salo et al 1994), and *M leprae* in ancient bones (Rafi et al 1994), thus showing the relevance of molecular biology studies to paleopathological research. In this paper PCR technique was tested to identify *T. cruzi* DNA in experimentally desiccated mouse tissue.

Materials and Methods

Four outbred 8 week old female mice were infected with 10^4 *T. cruzi* trypomastigote stage of the CL strain. Two noninfected mice of the same age and sex were used as controls. On the 4th, 8th, 14th, and 18th days of infection the parasitemia was measured by the Hoff method (Hoff 1974) to test the strain behavior, and all mouse infections were compatible for this parameter. On the 18th day of infection all the mice were killed, and the heart, skeletal muscle, liver, spleen, and pancreas of infected mice were put on a sheet of filter paper. Similar organ fragments of the control mice were also submitted to the same process. All the samples (infected and noninfected groups) were desiccated at 39° C for 90 days. After this period, when no weight variation occurred, fragments nearly 3 x 3 mm were removed from each organ and put in polypropylene tubes with guanidine-EDTA solution.

The method used to treat the samples prior to the PCR amplification has been previously described (Britto et al 1993). Briefly, the polypropylene tubes containing the guanidine-EDTA and organ fragments were immersed for 15 minutes in boiling water to break up the majority of the minicircle DNA molecules forming the kDNA network. After cooling to room temperature, two aliquots of 100 μl were taken from each sample, and extracted once with phenol-chloroform, then once with chloroform, prior to precipitation with two volumes of ethanol in 100 mM sodium acetate. The pellets obtained after centrifugation were each resuspended in 50 μl of distilled water. All amplifications were carried out in a final volume of 75 μl, using the hot-start procedure with physical separation of the primers and the DNA polymerase by solid paraffin layer. In this method, only the DNA polymerase and the oligonucleotides are in contact at high temperature, thus avoiding the synthesis of unspecific products (Chou et al 1992). The lower solution consists of 5 μl of 10x **Taq polymerase** reaction buffer (100 mM tris HCl, pH 8.3, 500 mM de KCl), 7.2 μl of a dNTPs mixture (10 mM each), 13.5 μl of 25 mM $MgCl_2$, 200 ng of the *T. cruzi* specific primers (#121:5'- AAATAATGTACGGG(T/G)GAGATGCATGA-3'; #122:5'-GG'TTCGATTGGGGGTTGGTGTAATATA-3'), and distilled water up to the final volume of 50 μl, in a thin walled reaction tube. After the addition of one ampliwax PCR Gem bead (Perkin-Elmer Cetus, Norwalk, CT), the tube was placed in an 80° C heating block to melt the paraffin bead, and then chilled to room temperature. After complete solidification of the wax barrier, 25 μl of the upper mixture were added to 7.5 μl of the DNA sample, 2.5 μl of 10x **Taq polymerase** and 2.5 units of **Taq polymerase** (Perkin-Elmer Cetus) and water to complete a final volume of 25 μl. The PCR was then immediately started using a DNA thermal cycle 480 (Perkin-Elmer Cetus) with the following conditions: two cycles at 98° C for 1 minute, and 64° C for 1 minute, 33 cycles at 94° C for 1 minute and 64° C for 1 minute, and a final extension at 72° C for 10 minutes (Wincker et al 1994). The DNA amplification products were submitted to electrophoresis in 2% agarose gel, and afterwards were stained with ethidium bromide for band detection.

Results

The amplification of all tissue samples (heart, skeletal muscle, liver, spleen, and pancreas) of the four infected mice using *T. cruzi* kDNA specific primers was positive, after electrophoresis and ethidium bromide staining. Results were checked using each sample studied, including one positive control (blood kDNA *T. cruzi*) and two negative tissue samples (negative control group), and one of only buffer. Negative controls present no specific bands, but the positive control showed specific banding pattern. Details of these results are shown in Fig. 1 (A, B and C).

Discussion

Fornaciari et al (1993) reported *T. cruzi* infection in archaeological material based on immunohistochemistry and electron microscopy studies. These samples were obtained from Peruvian mummies that presented with megacolon/megaesophagus, and were from areas where Chagas' disease is endemic. Although these pathologic entities are suggestive of *T. cruzi* infection, this finding occurs only in a small percentage of infections, varying with the geographic endemic area (Schenone et al 1980; Rothhammer et al 1985). That macroscopic parameter is therefore of limited value in paleopathological and paleoparasitological studies.
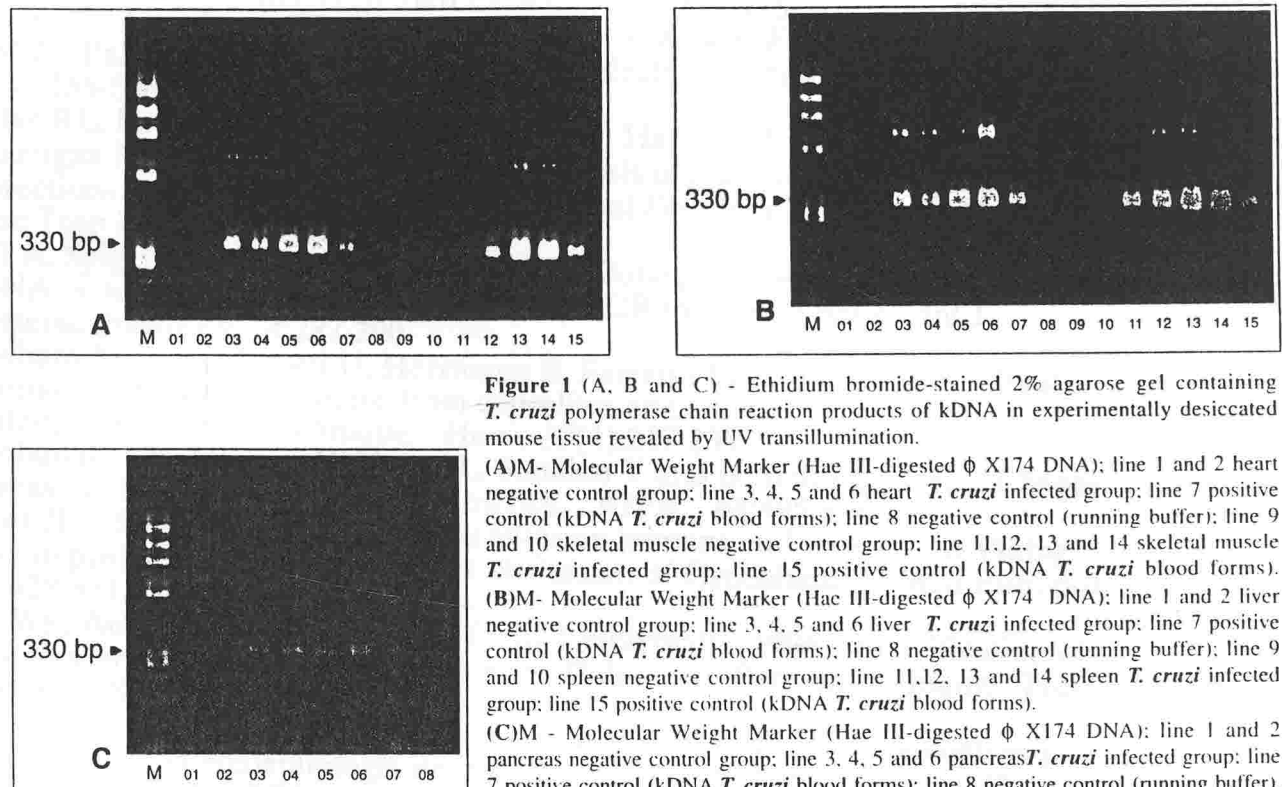
Figure 1 (A, B and C) - Ethidium bromide-stained 2% agarose gel containing *T. cruzi* polymerase chain reaction products of kDNA in experimentally desiccated mouse tissue revealed by UV transillumination.

(A)M- Molecular Weight Marker (Hae III-digested φ X174 DNA); line 1 and 2 heart negative control group; line 3, 4, 5 and 6 heart *T. cruzi* infected group; line 7 positive control (kDNA *T. cruzi* blood forms); line 8 negative control (running buffer); line 9 and 10 skeletal muscle negative control group; line 11,12, 13 and 14 skeletal muscle *T. cruzi* infected group; line 15 positive control (kDNA *T. cruzi* blood forms).
(B)M- Molecular Weight Marker (Hae III-digested φ X174 DNA); line 1 and 2 liver negative control group; line 3, 4, 5 and 6 liver *T. cruzi* infected group; line 7 positive control (kDNA *T. cruzi* blood forms); line 8 negative control (running buffer); line 9 and 10 spleen negative control group; line 11,12, 13 and 14 spleen *T. cruzi* infected group; line 15 positive control (kDNA *T. cruzi* blood forms).
(C)M - Molecular Weight Marker (Hae III-digested φ X174 DNA); line 1 and 2 pancreas negative control group; line 3, 4, 5 and 6 pancreas*T. cruzi* infected group; line 7 positive control (kDNA *T. cruzi* blood forms); line 8 negative control (running buffer).

The PCR technique may be used in different organ tissues, bone marrow included, offering a greater spectrum of research possibilities, apparently representing a useful investigative tool for infectious diseases in archaeological samples. This has been shown in mycobacterial infections (Spigelman & Lemma 1993, Salo et al 1994, Rafi et al 1994). It is interesting to note that, in spite of being desiccated, *T. cruzi* kDNA samples did not hinder the amplification by PCR in these experimental conditions. Similar results were demonstrated by Jones et al (1993) in inflammatory lesions of chronic cardiac Chagas' disease autopsy material. The preliminary data presented here, using experimentally desiccated *T. cruzi* infected tissues, represent the first step in the examination of South American mummified tissues for Chagas' disease. The technique used in these samples seems to be adequate to amplify *T. cruzi* kDNA in desiccated material, suggesting that it can also be used in samples of archaeological material.

Acknowledgements

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Reviewer's comment

This paper is important not only for the fine results achieved, but also for the principle it represents. How often have we seen paleopathologists jump in to apply a new technique to mummified tissue without knowing whether it really works under controlled, experimental conditions. Let this paper stand as a model.