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High correlation between Chagas' disease serology and PCR-based detection of *Trypanosoma cruzi* kinetoplast DNA in Bolivian children living in an endemic area

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Abstract: The detection of *Trypanosoma cruzi* kinetoplast DNA by polymerase chain reaction (PCR) amplification is a potentially powerful tool for the parasitological diagnosis of Chagas' disease. We have applied this technique in a field situation in Bolivia, where 45 children from a primary school were subjected to serological testing, buffy coat analysis and PCR diagnosis. 26 of the 28 serology-positive individuals were also positive by PCR. In addition, two serology-negative children gave a positive result by PCR, including one who was positive in the buffy coat test. These results suggest that PCR detection of *T. cruzi* DNA in blood can be a very useful complement to serology in Chagas' disease diagnosis in Bolivia.

Key words: Trypanosoma cruzi; Chagas' disease; Kinetoplast DNA; PCR diagnosis

Introduction

Chagas' disease, caused by the parasitic protozoan *Trypanosoma cruzi*, is an important public health problem in most countries of Latin America. Its direct diagnosis is difficult, due to the low concentration of parasites in the blood of infected persons. Because of this limitation, Chagas' disease diagnosis relies mainly on serological techniques. The sensitivity of these serological methods is generally high, but their use presents two main problems. First, the existence of cross-reactive epitopes between *T. cruzi* and other parasites circulating in the same geographical area may lead to false-positive results [1]. Second, the clinical status of a patient can be unlinked to his humoral response, as for example during the first weeks of an infection (when no serological reac-

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tion is yet observed) or after specific treatment (when an immune response can persist for years even if the treatment has been successful [2]). These considerations have justified the quest for a more efficient method of parasite detection in chagasic patients. The principal technique that has been tested for *T. cruzi* detection in blood samples is the polymerase chain reaction (PCR). Two main systems, leading respectively to the amplification of kinetoplast minicircle DNA [3–5] or of nuclear satellite sequences [6,7] have been described. Reconstitution experiments have suggested that these techniques are potentially able to detect a single parasite cell in 20 ml of blood [4,8]. However promising, these methods have yet to be validated in clinical samples originating from different geographical regions, due to the great variations in Chagas' disease incidence and clinical manifestations in the Americas. One large-scale study has been conducted in an endemic region of Brazil, showing that PCR can attain a high level of sensitivity in this situation [9]. The present work is aimed at testing the efficacy of PCR diagnosis of Chagas' disease in a radically different situation in Bolivia.

Materials and Methods

Patients and clinical samples

The individuals examined in this study belonged to two different groups. The first was composed of 45 children from the Mizque locality, Campero province, Cochabamba department, Bolivia. They were 5 to 8 years old, and were previously selected by a serological and parasitological (buffy coat) diagnosis for Chagas' disease when they were at school. The second was composed of 8 control individuals from Rio de Janeiro and La Paz who had never lived in endemic areas.

Ten ml of blood were collected from each individual, 5 ml of which were mixed immediately with an equal volume of 6 M guanidine HCl/0.2M EDTA for further processing for the PCR test [4]. The remaining 5 ml were used for serum preparation and buffy coat testing.

Serology and buffy coat tests

Serology was performed by indirect hemagglutination using the HEMAVE kit (Polychaco, Argentina), indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA). A patient was considered serology-positive when his serum reacted in at least two of the three tests. Buffy coat testing was performed in quadruplicate and observed by 2 independent people.

DNA preparation

The guanidine-EDTA-blood lysates were heated for 15 min in boiling water in order to shear the minicircle molecules that constitute most of the kDNA into moderately-sized pieces, and equalise their overall concentration [8]. One hundred μl was used for DNA preparation. After phenol-chloroform and chloroform extraction, the material was precipitated with ethanol. The pellet was resuspended in 50 μl of distilled water and stored at −20°C. All these and subsequent steps were performed in a laminar flow hood in a separate room not used for manipulation of amplified products, with dedicated micropipettes and filter-protected tips.

PCR conditions

The amplification reactions were performed in a volume of 75 μl using the 'hot-start' protocol with a solid paraffin barrier separating the Taq DNA polymerase from the oligonucleotides [10]. The lower phase consisted of 4 μl of the 10 × Taq polymerase buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.8 μl of a 10 mM dNTPs solution, 13.5 μl of a 25 mM $MgCl_2$ solution, 200 ng of *T. cruzi*-minicircle specific primers (5′-AAATAATGTACGGG(T/G)GAGATGCATGA 3′ and 5′ GGTTCGATTGGGGTTGGTG-TAATATA 3′ [11]), and water up to 40 μl, in a thin-walled reaction tube. An Ampliwax PCR Gem Bead (Perkin-Elmer) was added, melted by placing the tube at 80°C for 5 min, and solidified at room temperature. The upper phase consisted of 7.5 μl of the DNA sample, 3.5 μl of 10 × Taq DNA polymerase reaction buffer, 2.5 U of Taq DNA polymerase and water up to 35 μl. The PCR reaction was performed using 2 cycles at 98°C for 1 min and 64°C for 2 min, 33 cycles at

94°C for 1 min and 64°C for 1 min, and one extension step at 72°C for 10 min. The amplified products were electrophoresed in a 2% agarose gel and visualised by ethidium bromide-staining.

Results and Discussion

In the PCR assay used in this study, each blood sample to be tested was subjected to two independent DNA purifications, in order to avoid any problems arising from inhibition in any particular DNA preparation. Each PCR test included 5 samples in duplicate, one DNA preparation from an individual from a non-endemic area (also in duplicate), an amplification reaction without DNA (as a negative control) and an amplification reaction with DNA from a previously characterised chagasic patient (as a positive control). Typical results are shown in Fig. 1. In this way, we analysed 28 blood samples from serology-positive children living in the endemic area. Twenty-six of them (93%) were detected in our PCR assay, while only four of them had parasites detectable in buffy coat testing (Table 1). A previous study has indicated the high sensitivity PCR when compared to xenodiagnosis in detection of *T. cruzi* in blood samples collec in Brazil [9]. Until the advent of PCR, xenod nosis was considered to be the most sensi parasitological technique available for Cha

Table 1

Comparison of the PCR results of all individuals with se and buffy coat test results

Patient status	Patient no.	PCR	
		Positive	Negati
Bolivian children			
Serology-positive with positive buffy coat test	4	4	0
with negative buffy coat test	24	22	2
Serology-negative with positive buffy coat test	1	1	0
with negative buffy coat test	16	1	15
Non-chagasic individuals	8	0	8

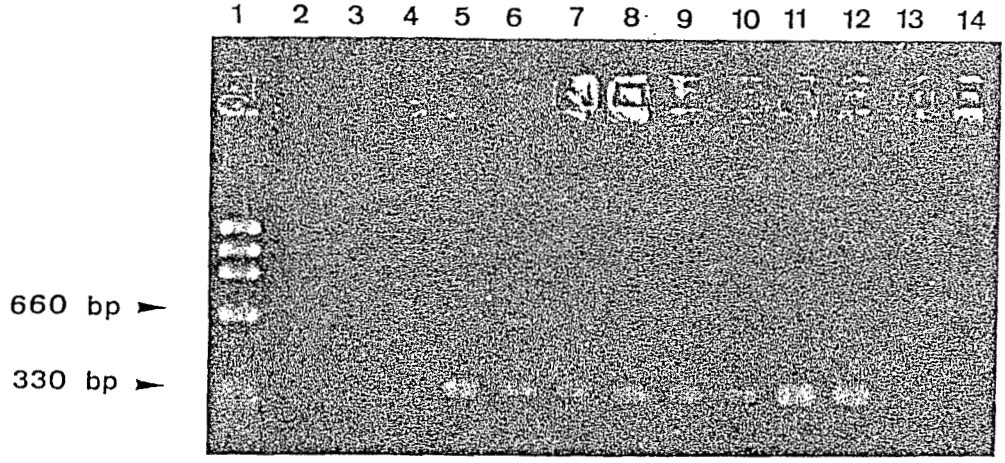

Fig. 1. Ethidium bromide-stained agarose gel from a PCR diagnosis experiment performed on 5 Bolivian children and o individual from a non-endemic area. All DNA preparations from blood samples and PCR amplifications were performed duplicate for each patient. Lane 1, ΦX174 DNA digested with *Hae*III; Lane 2, amplification reaction without added DNA; lane and 4, amplifications from a control individual; Lanes 5 and 6 to 11 and 12, amplification products from four Bolivian children w positive serology; lanes 13 and 14, amplifications from a serology-negative Bolivian child. Fifteen μl of the total reaction volu (75 μl) was loaded on this gel. The arrows indicate the expected products of the minicircle DNA amplification: a 330 bp ba corresponding to a single variable region and a 660 bp corresponding to a dimer.

disease diagnosis [12]. The results of the present study extend these findings to a different geographical area and suggest that PCR may find extensive applications in situations where other methods for parasitological diagnosis of Chagas' disease are currently being used.

Seventeen blood samples from serology-negative children were also submitted to our PCR assay, and 2 of them were positive (Table 1). Three arguments make the possibility of this result having occurred due to DNA contamination improbable. Firstly, we did not detect any contamination in 8 DNA preparations from control individuals processed in duplicate at the same time as the blood samples from the Bolivian children, while the two serology-negative, PCR positive samples gave an amplification in both of the duplicate DNA preparations. Secondly, the endemic region where these children live is considered to be an area of active transmission for Chagas' disease [13]. This renders the existence of young individuals who are serology-negative, but present a detectable parasitemia, explicable in cases of recently acquired infections, as the time necessary to mount a detectable humoral response is estimated to be several weeks [14]. Finally, one of the two serology-negative, PCR-positive children had *T. cruzi* cells in his blood as observed in buffy coat testing (Table 1). It should be noted that this result is consistent with the observation that parasitemia levels are generally high in the initial acute phase of Chagas' disease, when no antibody responses are yet detectable [15].

The high level of sensitivity and specificity of PCR detection of *T. cruzi* DNA in blood, observed in this and previous studies in an endemic region with very different characteristics, suggests that this technique will be a very valuable tool to study the evolution of Chagas' disease in patients in different epidemiological situations.

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