

PCR-based diagnosis for Chagas' disease in Bolivian children living in an active transmission area: comparison with conventional serological and parasitological diagnosis

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SUMMARY

A large field study has been performed in the Cochabamba region of Bolivia with the aim of comparing the polymerase chain reaction (PCR) with other diagnostic methods for Chagas' disease. The amplification of *Trypanosoma cruzi*-specific kinetoplast DNA sequences in blood samples was compared with classical serological methods, specific IgM detection and direct parasite visualization for 268 school children in a single village where Chagas' disease transmission is active. Of 113 children positive by classical serology or buffy coat examination, 106 were detected by PCR (sensitivity: 93.8%). We did not observe any significant difference of PCR sensitivity between initial (IgM and/or buffy coat positive) and indeterminate stage (only IgG positive) patients. Among the remaining 155 children unconfirmed as chagasic (who were either only IgM positive, IgG-, IgM-, and buffy coat -negative) only 1 case was PCR positive. This case may be due to DNA contamination, or to a very recent infection not detected otherwise, or to specific immune depression. These results show that PCR is a very sensitive parasitological test for Chagas' disease in active transmission regions. The future follow-up of the possibly infected patients who were only IgM-positive should clarify the interest of PCR and IgM tests in the detection of starting infections.

Key words: *Trypanosoma cruzi*, Chagas' disease, PCR diagnosis, serological diagnosis.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* is the aetiological agent of Chagas' disease, a very important public health problem in Latin America. It is transmitted by triatomine bugs during their blood meals. Albeit an important control programme has been started some years ago to eliminate the domiciliary species of triatomines, many regions in Latin America still have an active transmission cycle due to economic difficulties in implementing it (Moncayo, 1993). Consequently, sensitive methods for Chagas' disease diagnosis are particularly important in these poorest regions to precisely assess the rate of infection and the impact of future control programmes.

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The diagnosis of Chagas' disease relies primarily on serological techniques. However, these techniques present many problems of specificity (Camargo, 1992). An additional problem that occurs in regions of active transmission is the failure of the traditional serological methods, based on the detection of anti-*T. cruzi* IgG, to detect recent infections. To circumvent these difficulties, it has been suggested that serological diagnosis may be complemented by an efficient parasitological method. These methods include the xenodiagnosis, the haemoculture and the direct microscopical examination of fresh blood (Chiari *et al.* 1989). The specificity of these techniques is high, but unfortunately their sensitivities cannot be compared to that of serology, due to the low parasite concentration in the blood of chagasic patients, principally during the chronic phase of the disease. Recently, the definition of different PCR amplification methods for detecting low quantities of

T. cruzi DNA have led to the hope of a new, highly sensitive parasitological technique for Chagas' disease diagnosis (Moser, Kirchoff & Donelson, 1989; Sturm *et al.* 1989; Taibi *et al.* 1995).

A series of recent studies have focused on the use of PCR amplification of kinetoplast DNA sequences in the diagnosis of chronic chagasic patients in different regions of Brazil (Avila *et al.* 1993; Wincker *et al.* 1994*a*; Britto *et al.* 1995*a, b*; Junqueira, Chiari & Wincker, 1996). They have shown that this technique can be almost 100% specific, and that its sensitivity can reach 45–96.5% as compared with serology. Most importantly, direct comparisons were performed with xenodiagnosis or haemoculture during these studies. In every case, the PCR proved to be a markedly more sensitive method (Wincker *et al.* 1994*a*; Britto *et al.* 1995*a*; Junqueira *et al.* 1996). The main limit observed with PCR was that its sensitivity never reached that of serology. A semi-quantitative analysis of these results showed that this was due to the very low concentration of *T. cruzi* in the blood of chronic patients in some particular regions, and that this probably reflects the genetic constitution of the parasite strains circulating there (Britto *et al.* 1995*b*; Junqueira *et al.* 1996). It is therefore clear that the main use of PCR in Chagas' disease diagnosis is to complement serological methods.

Another application of PCR can be found in active transmission areas, where acute and chronic cases co-exist. As the most recently infected individuals generally have a high parasitaemia, but present a delay in raising their antibody response, it may be hypothesized that PCR could be useful in this situation. A preliminary study made with 45 children in Bolivia revealed a high correlation of PCR and IgG serology in these cases and confirms that PCR can detect chagasic individuals with a negative IgG serology (Wincker *et al.* 1994*b*). In the present work, we analysed a large number of children from this endemic area of active transmission, and compared the PCR and IgG serology results with other methods used to diagnose recent infections.

MATERIALS AND METHODS

Patients and sera

The individuals examined in this study were children (free of treatment) living in Mizque village located in a highly endemic region of Bolivia (Cochabamba department). The blood samples were collected at the same time, mainly in the elementary school. The average age was 7.7 years old ranging from 1 to 15 years old. None of these children presented clinical symptoms of acute-phase illness. Ten ml of blood were collected from each child and 5 ml of it were immediately mixed with an equal volume of 6 M guanidine HCl/200 mM EDTA, pH 8 (Avila *et al.* 1991). The remaining blood was processed for

parasite visualization in the buffy coat (BC test; La Fuente, Saucedo & Urgel, 1984) and serum preparation.

Serological techniques

Specific anti-*T. cruzi* IgG were detected in 4 assays. First, haemagglutination (HEMAVE test, Polychaco, Buenos Aires, Argentina) was carried out in the field. Second, immunofluorescence was performed according to Alvarez, Cerisola & Rohweder (1968) at the IBBA, La Paz, using *T. cruzi* epimastigotes fixed by 1% glutaraldehyde, and a mouse FITC-labelled anti-human IgG (H+L) conjugate (Biosys, Compiègne, France) diluted 1/200. A detection limit of 1/32 was selected. Finally, enzyme-linked immunosorbent assay (ELISA) was performed in 2 different laboratories (IBBA, La Paz and Institut Pasteur, Paris) according to previous studies (Brenière *et al.* 1984; Aznar *et al.* 1995). The sheep peroxidase-conjugated anti-human IgG (H+L) used in IBBA, was from Biosys (Compiègne, France) and used at a dilution of 1/2500. The goat F(ab')₂ alkaline phosphatase-conjugated anti-human IgG used in Institut Pasteur was from Caltag (San Francisco, USA) and diluted 1/4000. Sera were diluted 1/200 in both cases and the limit extinction values of ELISA and optical density ratio were 0.2 and 1.00 respectively. The diagnosis was based on positivity or negativity of 3 out of 4 tests. Moreover, specific IgM antibodies were detected by ELISA technique in the Institut Pasteur using the *T. cruzi* antigen as previously described (Aznar *et al.* 1995). The antigen was diluted to a final concentration of 2.5 µg/ml in PBS (pH 7.2). It was successively incubated with sera diluted 1/200 and goat F(ab')₂ peroxidase-conjugated anti-human IgM (Diagnostic Sanofi Pasteur) diluted 1/2000.

DNA extraction and PCR conditions

The tubes containing the guanidine-EDTA-blood lysates were immersed for 15 min in a boiling water bath to split the kDNA network (Britto *et al.* 1993). They were cooled to room temperature, and two 100 µl aliquots were taken for DNA preparation. These aliquots were extracted once with phenol-chloroform and once with chloroform, and then precipitated with 2 volumes of ethanol in 100 mM sodium acetate. After centrifugation, the pellets were resuspended in 50 µl of distilled water.

The amplifications were carried out in a final volume of 75 µl using the hot-start procedure with a solid paraffin barrier separating the oligonucleotides and the *Taq* polymerase (Chou *et al.* 1992). The lower solution consisted of 5 µl of *Taq* polymerase reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 7.2 µl of a dNTPs mixture (10 mM each), 13.5 µl of 25 mM MgCl₂, 200 ng of the *T. cruzi*-specific primers (5' AAATAATGTACGGG(T/G)-

Table 1. PCR-based diagnosis: comparison with conventional tests (BC = Buffy coat test; IgG = specific anti-*Trypanosoma cruzi* immunoglobulins G; IgM = specific anti-*T. cruzi* immunoglobulins M; confirmed chagasic patients are patients with BC and/or IgG tests positive.)

Patients (number)	IgG	IgM	PCR
Confirmed chagasic (113)			
With positive BC test			
No. positive	9	3	11
No. negative	3	9	1
With negative BC test			
No. positive	101	15	95
No. negative	0	86	6
Possibly infected (10)			
No. positive	0	10	0
No. negative	10	0	10
Uninfected (145)			
No. positive	0	0	1
No. negative	145	145	144

GAGATGCATGA 3' and 5' GGTTTCGATTGG-GGTTGGTGTAATATA 3'), and water to a final volume of 50 μ l in a thin-walled reaction tube. The upper phase was made of 7.5 μ l of the DNA sample, 2.5 μ l of 10 \times *Taq* polymerase buffer, 2.5 μ l of *Taq* DNA polymerase and 12.5 μ l of water. The PCR reaction was immediately started using a DNA thermocycler 480 (Perkin-Elmer) with the following parameters: 2 cycles at 98 °C for 1 min and 64 °C for 1 min, 33 cycles at 94 °C for 1 min and 64 °C for 1 min, and a final extension at 72 °C for 10 min. The same protocol allowed the amplification of the human β -globin sequences with the primers 5' ACA-CAAACCTGTGTTTCACTAGC 3' and 5' CAACT-TCATCCACGTTCCACC 3'. The PCR products were analysed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

Prevalence of Chagas' disease in the endemic area

The present study was conducted in a rural setting in the Cochabamba region where no insect control programme has been implemented yet. A total of 268 school children (1–15 years old) was randomly selected to be diagnosed for Chagas' disease. Before performing the PCR diagnosis test, we first applied a serological diagnosis aimed at detecting IgG in the blood using 4 different techniques (described in the Materials and Methods section). Two further tests were performed on all children to detect possible recent infections. First, a direct examination for parasites after concentration in the buffy coat was performed. Second, an IgM-specific detection reaction was made at the Institut Pasteur. Table 1 summarizes the results. One-hundred and thirteen children (42.2%) were positive in at least 3 of the 4 tests detecting specific IgG and/or positive in direct

parasitological diagnosis (buffy coat), and were considered infected by *T. cruzi* (we will refer to them as 'confirmed chagasic patients' from now). This result shows the high prevalence and the active transmission of the disease in this region. Among the confirmed chagasic patients group, 12 patients presented a positive buffy coat and 15 a positive specific IgG reaction associated to a positive specific IgM reaction (Table 2) with a negative buffy coat, and were considered recently infected (23.9%). The other 86 confirmed chagasic patients were classified as in an indeterminate state of the disease. Furthermore, 10 patients presented only a positive specific IgM detection with a negative specific IgG reaction and a negative buffy coat and were considered as possible *T. cruzi* infections (3.7% of the entire population). We note that in this last group the majority of the patients present a high rate of IgM antibodies (Table 2). The other 145 patients with negative serological and negative buffy coat were considered free of *T. cruzi* infection.

PCR diagnosis

All 268 children previously examined were further submitted to a PCR diagnosis test. This test used specific amplification of minicircle sequences and was previously shown to be sensitive enough to detect 1 parasite in 20 ml of blood (Britto *et al.* 1993). A typical result for a series of PCR tests, including the positive and negative controls systematically included, is presented in Fig. 1. We found that, out of 113 chagasic patients, 106 were PCR positive (93.8%). Moreover, we did not observe significant differences in the PCR tests between the recent infections and the indeterminate group (88.8% and 95% of positivity respectively, Chi 2 = 1.47, $P > 0.05$). In the group of non-infected children, the PCR test was positive in 1 case (see

Table 2. Specific IgM antibodies in the blood of Bolivian children (BC = Buffy coat test; IgG = specific anti-*Trypanosoma cruzi* immunoglobulins G.)

Specific IgM values	Patients status				Total no. of cases
	BC +		BC -		
	IgG +	IgG -	IgG +	IgG -	
OD ratio < 1.00	8	1	86	145	240
1.00 < OD ratio < 1.10	1	1	2	4	8
OD ratio > 1.10	0	1	13	6	20

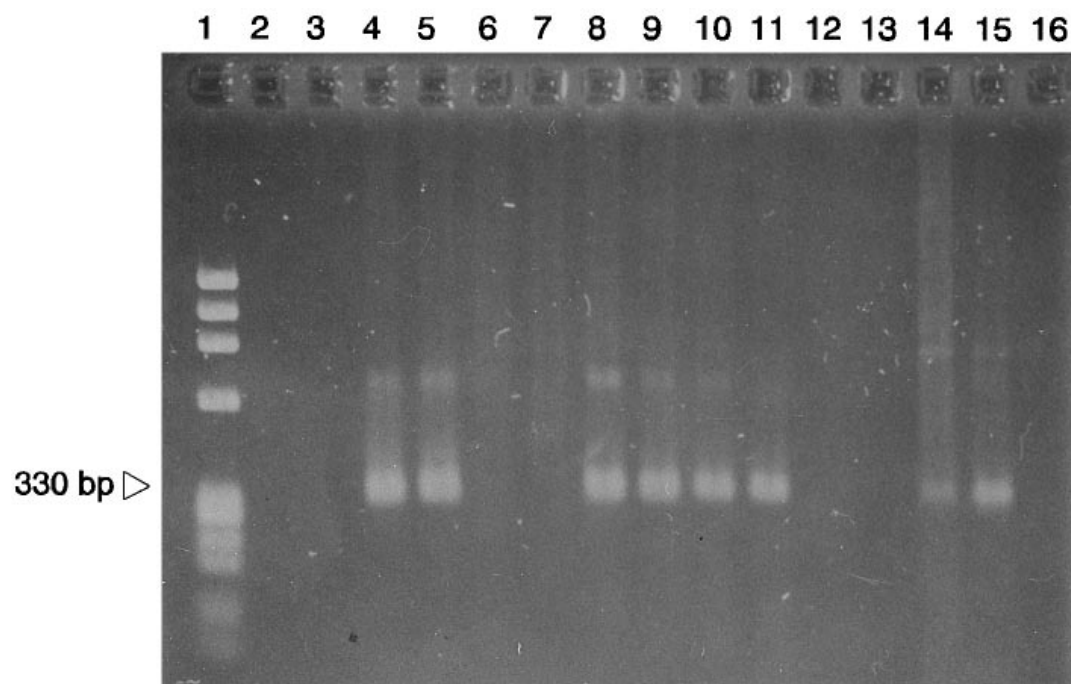


Fig. 1. Typical PCR diagnosis experiment. Lane 1, molecular weight marker (Φ X174 DNA-*Hae* III digested); lanes 2 and 3, negative controls (normal individual); lanes 4 and 5-12 and 13, Bolivian children; lane 14, positive control (confirmed chagasic patient with low parasitaemia); lane 15, positive control (confirmed chagasic patient with high parasitaemia); lane 16, negative control of the reaction (no DNA added). All reactions except the positive controls were performed with duplicate DNA preparations, using 35 amplification cycles in a volume of 75 μ l. Fifteen μ l were applied to each gel lane, and the results were revealed using U.V. transillumination after ethidium bromide staining. All the negative DNA preparations were checked for possible inhibition using a human β -globin amplification.

Discussion section). Finally, the PCR test was always negative in the possibly infected group (only IgM positive).

It is worth noting that the PCR test reached a high level of accordance only with the specific IgG test (96.6%). Out of 12 positive buffy coats, 11 PCR tests were positive (91.6%). Moreover, out of 28 patients presenting positive IgM, 16 had a positive PCR test (57.1%). We also observed that the values of positive IgM antibodies ranged from 1.02 to 2.63 (optical density ratio) and that 20 patients present an IgM optical density ratio of > 1.10 (71.43%, Table 2). We did not observe significant differences in the PCR test between these 2 groups presenting low and high levels of IgM antibodies.

When comparing the results obtained in the confirmed chagasic patients group, we found that out

of 18 IgM positive cases, 14 were IgG and PCR positive, while 2 were only PCR positive and 2 only IgG positive.

DISCUSSION

The present study was intended to test the interest of PCR diagnosis for Chagas' disease in a situation of active transmission. It complements previous studies carried out in areas where transmission was stopped. In these studies the sensitivity of PCR, when compared to serology (IgG detection), was found to be dependent on the level of parasitaemia observed with the strains circulating in each area. It reached 45, 60 and 96.5% for 3 Brazilian regions where the circulating strains gave rise to low, intermediate and high parasitaemia, respectively (Britto *et al.* 1995b;

Junqueira *et al.* 1996; Wincker *et al.* 1994a). The results of the present study demonstrate that we can expect a high degree of sensitivity in an active transmission area. This result may be related to a high frequency of re-infection, to the low immune status of the patients (perhaps in relation with their poor living conditions) or to the genetic constitution of the *T. cruzi* strains circulating in the area. It is worth noting that in the 3 Brazilian areas mentioned above, the transmission had been stopped and therefore the hypothesis of frequent reinfections was not applicable. On the contrary, it is known that experimental infections with different clones of *T. cruzi* induced different patterns of parasitaemia (Sanchez *et al.* 1990; Laurent, 1994). This supports the hypothesis of a variation of parasitaemia rates in human relating in part to the genetic constitution of *T. cruzi* clones. Further work is needed to address these points.

Taken together, all these previous results indicate that PCR, although not sufficiently sensitive to be used for Chagas' disease diagnosis alone, is the best complementary technique to serology for this purpose. Its very high specificity is also an advantage for the diagnosis of difficult cases, e.g. patients with a borderline response in serology. With the data obtained in the present work, we can discuss for the first time the interest of the PCR test for the early diagnosis of Chagas' disease. The high specificity and sensitivity of PCR are particularly important for recent infections, as the drugs presently in use for Chagas' disease treatment are much more efficient in the acute phase than in the chronic phase (De Castro, 1993). A rapid and sensitive diagnosis is therefore needed to decide the start-up of the treatment. Acute stage or initial infection is commonly defined by the presence of circulating parasites evidenced by direct parasitological examination which is difficult and depends on the microscopist. An alternative is the detection of specific anti-*T. cruzi* IgM antibodies, as these appear 3–4 days after infection, present a peak around 15–20 days later and decrease progressively (Braun & Titto, 1985). Specific IgG antibodies are detectable 10–15 days after infection (Vattuone, Szarfman & Gonzalez-Cappa, 1978; Schmunis *et al.* 1980). Consequently, patients presenting IgM antibodies associated or not with specific IgG, are considered in the initial stage of the infection. In this last group of patients, the sensitivity of the PCR test was low (57.1%). This may indicate that PCR is of low interest for the diagnosis of these cases or, alternatively, that the specificity of the IgM detection may be low and had led to some false-positive results.

The single case of a PCR-positive individual who remained negative with all other techniques may be explained either by DNA contamination, or a very recent infection not detected otherwise or a patient presenting specific immune suppression (Brenière *et*

al. 1984). DNA contamination is extremely unlikely, as the PCR test was carried out in duplicate after 2 independent DNA extractions and was positive in both assays. A long-term follow-up of this individual will be necessary to discriminate among these hypotheses.

The analysis of the different tests obtained among the 268 patients permits proposal of a kinetic model for specific antibodies and parasitaemia: first synthesis of IgM (few days after infection) followed by synthesis of IgG and later increase of parasitaemia. This model explains 264 cases out of 268. In particular, we observed 10 patients with positive IgM alone, 15 patients with positive IgG and IgM but with negative direct parasitaemia (buffy coat) and finally, out of 12 patients with a positive buffy coat, 8 presenting already IgG positivity without IgM positivity. If the rise in parasitaemia occurred before or simultaneously with IgG synthesis the last 2 groups would remain difficult to explain. According to this model, the PCR test would fail to diagnose only the earliest cases of infection (positive IgM antibodies alone). This work must be complemented by a follow-up of young populations presenting IgM antibodies to assess the interest of this marker compared with PCR in detecting early cases of Chagas' disease.

An overt advantage of the PCR detection of *T. cruzi* in blood over other methods of parasite detection as buffy coat examination, haemoculture or xenodiagnosis, is the gain of sensitivity, consistently documented in all studies performed to date. This is particularly important for epidemiological surveys, as the samplings carried out with classical methods were probably biased towards the fastest-growing strains isolated after a culture step. With the PCR test used in the present study, an amplified product corresponding to the minicircle content of a whole infective population of parasites can be obtained. Recent studies have shown that these amplified products could be useful as hybridization probes to study the strains infecting a particular patient (Britto *et al.* 1995b) or to look at the triatomine population in which a particular strain is circulating (Brenière *et al.* 1992, 1995). A future development of the present work would be the application of such hybridization techniques to study the *T. cruzi* strains involved in current transmission.

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