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Detection of *Trypanosoma cruzi* in untreated chronic chagasic patients is improved by using three parasitological methods simultaneously

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Objectives: This study compared three parasitological methods applied simultaneously in individuals with untreated chronic Chagas' disease in order to determine their individual and combined performances.

Methods: From a total of 100 chronic chagasic patients from endemic areas of Chile, with informed consent, we extracted 2 mL of peripheral venous blood for PCR (PCR-B) and applied two xenodiagnosis (XD) boxes with seven uninfected *Triatoma infestans* nymphs each for microscopic examination and PCR of faecal samples of the triatomines fed on each patient (PCR-XD). The PCR-B and PCR-XD reactions were performed with oligonucleotides 121 and 122, which anneal to the four constant regions of the minicircles of *Trypanosoma cruzi* kinetoplasts. The 330 bp PCR product was analysed by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Results: PCR-B detected *T. cruzi* in 58% of the cases, while PCR-XD proved to be more sensitive than XD (67% versus 14%, respectively) ($P=0.0001$). There was no difference between the detection power of PCR-B and PCR-XD ($P=0.222$). The percentage detected as positive was much greater when the three tests were considered (84%) ($P=0.00001$).

Conclusions: The simultaneous application of more than one technique for the parasitological diagnosis of Chagas' disease in untreated individuals increases the possibility of detection of *T. cruzi*.

Keywords: Chagas' disease, xenodiagnosis, PCR

Introduction

Chagas' disease is the most prevalent human parasitosis in Latin America, where there are between 15 and 16 million infected persons and 75–90 million exposed to infection. At present, Chagas' disease constitutes a global public health problem due to the flow of migration to developed countries from the Latin American countries where Chagas' disease is endemic. In Chile, the endemic–enzootic area is distributed in rural and peri-urban areas of the eight most northern administrative regions and the Metropolitan Region; there are about 150000 infected persons. There is a current consensus that Chagas' disease must be treated in all its stages; nevertheless, treatment efficacy is hampered by the lack of reliable criteria of cure for the chronic cases.[1,2] PCR has become the most promising tool for parasitological studies before therapy and in follow-up after chemotherapy, showing greater sensitivity than the traditional parasitological tests such as xenodiagnosis (XD) and blood culture.[2] PCR can detect 2.5 parasite genome equivalents/mL of blood in agarose gels or 0.25 parasite genome equivalents/mL of blood after Southern hybridization. Thus a 2 mL blood sample containing at least one intact parasite or 0.5 genomic equivalents should be positive in a PCR reaction targeted to the 330 bp minicircle fragment of *Trypanosoma cruzi* kinetoplastid (kDNA).[3] In this study, PCR of peripheral blood (PCR-B), XD and PCR of faecal samples of triatomines fed on chronic chagasic patients (PCR-XD) were applied in order to determine their individual and global performances in untreated infected individuals.

Methods

The patients were 100 chronic chagasics (89 women and 11 men) from different areas of the provinces of Choapa and Combarbalá, IV Region, Chile, with ages between 17 and 72 years (average 39 years). All were

detected in epidemiological screening performed in those endemic areas between 2009 and 2010 with informed consent, approved by the Ethics Committee of the Faculty of Medicine, University of Chile, and volunteered to participate in the study. *T. cruzi* infection was determined by indirect immunofluorescence (IIF) and ELISA in our laboratories. A sample of 2 mL of peripheral blood was mixed with 6 M guanidine-HCl/0.1 M EDTA buffer (pH 8.0) and incubated at 98°C for 15 min to break the minicircles of *T. cruzi* kinetoplasts. DNA extraction was performed on 200 μL of guanidine-mixed samples, using the FavorPrep Blood Genomic DNA Extraction kit (Favorgen Biotech) according to the manufacturer's instructions and maintained at −20°C until use. In parallel, XD was applied using two cylindrical wooden boxes, each containing seven uninfected third instar *Triatoma infestans* nymphs. The insects were allowed to feed for 20–30 min on the arm of each volunteer. Microscopic examination of faecal samples was performed 30, 60 and 90 days after feeding triatomines.[4] For PCR-XD, the faecal samples of all the triatomines obtained after 30, 60 and 90 days were mixed with PBS 7.2, incubated at 98°C for 15 min and centrifuged at 4000 rpm for 3 min. The supernatants were pooled for DNA extraction and maintained at −20°C until use. The PCR-B and PCR-XD were performed in triplicate with oligonucleotides 121 and 122, which anneal to the four constant regions present in minicircles of *T. cruzi*,[4] to obtain 320 bp amplicons. The PCRs were performed in a final volume of 20 μL containing 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer (121 and 122) and 1 U of GoTaq DNA polymerase (Promega). The amplification programme was performed in a TC 412 thermal cycler (Techne), and included an initial denaturation at 98°C for 1 min and 64°C for 2 min; 33 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 10 min. Each experiment included 5 μL of Bench Top 100 bp DNA ladder (Promega); a PCR control that contained water instead of DNA; DNA of non-chagasic patients and a positive control, the purified DNA *T. cruzi* Tulahuén strain. Amplification products were analysed by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide (Figure 1).[5] Statistical analysis was performed by means of the Cochran Q test to compare whether the three methods had the same percentage of positives. The McNemar *a posteriori* test was used to identify which methods had different percentages of positives.

Results and discussion

Chronic chagasic patients treated with benznidazole and evaluated in extended follow-up demonstrated a reduced risk of progression of cardiac alteration in comparison with a non-treated group.[6] On the other hand, chronic chagasic patients treated with nifurtimox or benznidazole in an extended follow-up demonstrated that the drugs had a beneficial effect on the evolution of the disease in at least 37% of the cases.[7] From this perspective, one of the most important challenges in the evaluation

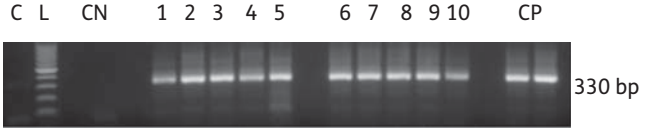

Figure 1. Ethidium bromide stained 2% agarose gel of amplified products of *T. cruzi* minicircle kinetoplastid PCR products from samples of peripheral blood (PCR-B) and faecal samples of triatomines fed on untreated chronic chagasic patients (PCR-XD). C, control PCR mix; L, 100 bp ladder; CN, DNA non-chagasic individual; Lanes 1–5, DNA peripheral blood of chronic chagasic patients (PCR-B); Lanes 6–10, DNA in faecal samples of triatomines fed on chronic chagasic patients (PCR-XD); CP, DNA *T. cruzi* Tulahuén strain.

Table 1. Detection of *T. cruzi* by xenodiagnosis (XD), PCR in peripheral blood (PCR-B) and PCR-XD in faecal samples of triatomines fed on 100 untreated chronic chagasic patients

	Results			
Parasitological condition	PCR-B	XD	PCR-XD	Patients (%)
	−	−	−	16
	+	−	+	32
	−	+	+	5
	−	−	+	21
	+	−	−	17
	+	+	+	9
Positive	58	14	67	84
Negative	42	86	33	16
Total	100	100	100	100

PCR-B=PCR-XD ($P=0.222$).
PCR-B versus XD and PCR-XD versus XD: $P=0.0001$ (statistically significant difference).
PCR-XD versus 84%: $P=0.0001$ (statistically significant difference).

of the chemotherapeutic efficacy in chronic Chagas' disease is the establishment of trustworthy cure criteria.[1,2] In the chronic period of the illness the parasitaemia is low, sub-patent and fluctuating, therefore simultaneous application of different parasitological techniques can increase the possibility of demonstrating *T. cruzi* circulating in chronic chagasic patients, who will then receive specific treatment. Nowadays, XD is used only by laboratories that have a bug nursery. It can be useful in cases of borderline serological results and for evaluation of the treatment of Chagas' disease and HIV co-infection.[8] *T. infestans*, the natural vector of *T. cruzi* that we used for XD, is fundamental for recovery of the biological material for PCR-XD. Our study demonstrated that PCR-XD (67%) was more sensitive than XD (14%) ($P=0.0001$) and increased the sensitivity of detection of *T. cruzi* by 53% (Table 1). *T. cruzi* detection by PCR-B was 58%, with a total of 84% positives when the three techniques were applied simultaneously ($P=0.00001$). Castro *et al*. (2002) compared two tests in three serial blood samples of 60 untreated chronic chagasic patients, demonstrating 70% positives for blood culture and 86.7% positives for PCR in samples obtained over a period of 1 year. The greater percentage obtained with PCR may be explained by the higher sensitivity of the test and by its application in serial samples, which allow the detection of circulating *T. cruzi* that could not be detected in one sample.[9] The usefulness of serial samples to improve the sensibility of PCR was reinforced by Araujo *et al*. (2002), who demonstrated in dogs infected with *T. cruzi* an increase in positives from 67% to 100% when a second sample was taken into account.[10] The results suggest that the parasitological condition of Chagas' disease may be determined by PCR-B in successive blood samples or simultaneous tests.

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Transparency declarations

None to declare.

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