Usefulness of PCR for monitoring benznidazole response in patients with chronic Chagas’ disease: a prospective study in a non-disease-endemic country

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Received 5 February 2010; returned 17 March 2010; revised 4 May 2010; accepted 9 May 2010

Objectives: This prospective study focused on the evaluation of antiparasitic therapy in chronic Chagas’ disease, monitored by PCR.

Patients and methods: One hundred and eighty-one patients, all seropositive for Trypanosoma cruzi infection, underwent specific chemotherapy with benznidazole. Twelve patients were classified as young (2–19 years), 122 patients were classified as adults (20–39 years) and 47 patients were classified as seniors (≥40 years). In total, 116 (64%) were asymptomatic and 65 (36%) were symptomatic. Patients were monitored for 90, 150 and 420 days after treatment by PCR and serology.

Results: Before treatment, 68% had a positive PCR result. PCR positivity was significantly more frequent in patients aged 2–19 years (P = 0.0007) and in short-term immigrants (P = 0.0076). No differences in PCR sensitivity were observed between samples of patients with chronic symptomatic or indeterminate Chagas’ disease. All patients presented an early conversion of PCR results 90 days post-treatment. However, at the end of the follow-up period PCR had become positive in four patients (4 out of 58 patients; 6.9%) who had strictly adhered to the treatment and in one who did not complete the treatment. Only one patient showed an alternating PCR during follow-up.

Conclusions: We conclude that PCR is a useful tool for the early detection of parasite susceptibility to benznidazole and for the post-treatment parasitological follow-up of patients with chronic Chagas’ disease.

Keywords: Trypanosoma cruzi, kinetoplast DNA, parasitological follow-up

Introduction

Chagas’ disease, or American trypanosomiasis, is a parasite zoonosis caused by Trypanosoma cruzi that is endemic in 21 countries of Latin America. The disease affects 8 million people globally.1 Chagas’ disease is a health problem not only in Latin America, but also in non-disease-endemic countries that receive immigrants.2,3 Of all the European countries, Spain receives the largest number of immigrants from Latin America (~2 million legal immigrants).3 The diagnosis and treatment of infected persons represent a challenge for receiving countries because health services are not accustomed to managing it. Two drugs have been used for the treatment of Chagas’ disease—the nitrofuran nifurtimox and the nitroimidazole benznidazole—although their effectiveness varies according to the phase of the disease and toxic side effects are frequent. Nevertheless, cure evaluation is the most complex aspect of treatment. After chemotherapy, the cure criterion for individuals with chronic Chagas’ disease is the persistence of negative serological and parasitological results,5 although seroconversion usually occurs several years after treatment.6 The parasitological response is usually monitored by microscopic examination of blood after parasite concentration following the Strout method, haemoculture or xenodiagnosis, all of which lack sensitivity.7 In an effort to obtain more sensitive assays, a PCR strategy has been developed to detect T. cruzi DNA in blood samples from patients with chronic Chagas’ disease. Thus, primers have been designed for the amplification of nuclear and kinetoplast T. cruzi DNAs, both of which contain many repetitive sequences that are highly suitable for PCR detection. In fact, PCR of kinetoplast DNA is able to detect the equivalent of a single parasite cell in 10 mL of whole blood.8,9
The purpose of the present study was to use PCR to assess the treatment efficacy in a well-characterized cohort of 181 patients with chronic Chagas’ disease diagnosed at the Unit of Tropical Medicine (UTM) of the Virgen de la Arrixaca Hospital in Murcia (Spain), from January 2007 through June 2009.

Methods

Subjects and data collection

This prospective follow-up study assessed PCR as a diagnostic and post-treatment parasitological follow-up tool in patients with chronic Chagas’ disease who were treated with benznidazole.

Among 418 cases of Chagas’ disease diagnosed in the UTM, from January 2007 through June 2009, a cohort of 181 patients with chronic Chagas’ disease was examined prospectively. Most patients were from Bolivia (176; 97.2%) with 5 (2.8%) from other Latin American countries such as Paraguay, Ecuador, El Salvador and Brazil. The mean (±SD) age was 33 ± 11 years and the median age was 32.

Diagnosis of T. cruzi infection was made according to WHO guidelines.5 Two serological tests with different antigens—an indirect immunofluorescence assay (Inmunofluor CHAGAS Kit; Biocientifica S.A.) and an ELISA (T. cruzi ELISA test system; Ortho Clinical Diagnostic, USA)—were used, following the manufacturers’ instructions. Patients with positive results for both serological tests were considered to be infected by T. cruzi.

The analyses were adjusted for age, clinical status and years of residence in Spain. Confirmed cases were interviewed, clinically studied and treated. To define the clinical groups, all patients had an electrocardiogram, chest radiography and echocardiography. Cardiac patients were classified according to the clinical classification of Kuschnir et al.7 Upper gastrointestinal transit and opaque enema were performed to classify digestive involvement. Clinical symptoms, such as dyspnoea as a result of effort or at rest, paroxysmal nocturnal dyspnoea, orthopnoea, lower extremity oedema, dysphagia, gastrointestinal reflux and constipation, were considered. Asymptomatic patients were those who had no evidence of cardiac or digestive involvement.

Of 181 patients with chronic Chagas’ disease, 116 (64%) were asymptomatic and 65 (36%) were symptomatic. In total, 20 (11%) had only cardiac disorders, 27 (15%) had both cardiac and digestive disorders and 18 (10%) had only digestive disorders.

According to their age, patients were stratified as follows: 12 patients were classified as young (2–19 years); 122 patients were classified as adults (20–39 years); and 47 patients were classified as seniors (≥40 years).

According to years of residence in Spain, patients were classified into one of two groups: 79 were included in a short-term group (<3 years); and 102 were included in a long-term group (≥3 years).

Indirect immunofluorescence

To analyse the serum titres, indirect immunofluorescence assays were performed using the Immunofluor CHAGAS Kit, according to the manufacturer’s instructions. Serum dilutions (1/40–1/10240) in PBS were incubated for 30 min at room temperature with the total antigen pre-adsorbed onto the slide surface. Unbound immunoglobulins were removed by washing the slides twice with PBS. Following incubation with fluorescein-labelled anti-human IgG, IgA and IgM conjugate for 30 min at room temperature, unbound conjugate was removed by two washes with PBS. Slides were mounted with buffered glycerine, pH 9.5, and observed under a fluorescence microscope. Positive chagasic and negative controls were used. The cut-off value for indirect immunofluorescence was 1/40.

DNA extraction

Blood samples (10 or 2 mL) collected from T. cruzi-infected adults or infants, respectively, were immediately mixed with one volume of 2× lysis buffer containing 6 M guanidine hydrochloride and 200 mM EDTA, pH 8, boiled for 15 min and stored at 4°C. DNA was extracted from blood using the Maxwell 16 Blood DNA Purification Kit (Promega Biotech Iberica) using 400 μL of sample according to the manufacturer’s instructions.

PCR amplification

PCR detection of the 330 bp variable regions of the T. cruzi kинетопласт mini-circle genome was carried out as described by Gomes et al.10 with slight modifications. Briefly, 200 ng of each sample was added to a final volume of 75 μL containing 0.2 mM dNTP mixture (Roche), 200 ng each of T. cruzi-specific primers 121 (5′-AAATAATGACGGGAGATGATGA-3′) and 122 (5′-GTTTCATGGGGTTTGCAGAATATA-3′), 2.5 U of Taq DNA polymerase (Roche), 7.5 μL of 10× Taq DNA polymerase buffer and 2.5 mM MgCl2. The cycling programme included an initial denaturation step at 95°C for 5 min, 40 amplification cycles at 94°C for 1 min, 64°C for 1 min and 72°C for 1 min and a terminal extension at 72°C for 10 min. A similar protocol was used as a PCR internal control for amplification of ribosomal DNA with the primers REV (5′-GACGGTATCTGATGCTTC-3′) and HUF (5′-GGACCGCTTGATACCG-3′). Negative controls were included in each PCR.

Gel electrophoresis was performed using 2% agarose gels and TBE buffer (44.5 mM Tris/44.5 mM boric acid/1 mM EDTA, pH 8.4) in the presence of 0.5 μg/mL ethidium bromide. A 100 bp (Invitrogen) ladder was used as a standard. Gels were analysed using a UV transilluminator. Blood extraction, DNA purification, cycling and gel electrophoresis procedures were carried out in separate laboratory working areas to avoid carry-over amplion contamination.

Benznidazole treatment

All patients (181) were treated orally with benznidazole, 5–7 mg/kg of body weight per day in paediatrics and 100 mg three times a day in adults, for 60 days.11 The cumulative dose did not exceed 18 g of body weight per day in paediatrics and 100 mg three times a day in adults. Of those, 57 patients (57/181; 31.5%) were treated with benznidazole to prevent some side effects such as polyneuritis and depression of bone marrow.12 Of these, 57 patients (57/181; 31.5%) presented with adverse reactions, some requiring symptomatic treatment. The side effects of benznidazole were cutaneous hypersensitivity [48/181 patients (26.5%), asthma [1/181 patients (0.6%)], gastrointestinal intolerance such as nausea, abdominal pain, vomiting and diarrhoea [4/181 patients (2.2%)], neurological [3/181 patients (1.7%)] and migratory arthritis [1/181 patients (0.6%)]. A total of 11 patients did not complete the treatment programme. In two cases treatment was not correctly observed and nine patients (9/181; 5%) had to suspend the treatment due to the side effects. Of the nine patients who had to interrupt the treatment, all did so because of dermatitis due to hypersensitivity.

Peripheral blood samples were obtained for serology and PCR at the time of diagnosis and ~90, 150 and 420 days post-therapy to evaluate the outcome of treatment. A flow diagram describing the number of patients included in the different phases of the follow-up is shown (Figure 1).

Statistical analysis

The χ2 test was used to compare qualitative variables. Relationships were considered significant if P < 0.05. All statistical tests were performed with SPSS 15.0 and G-StaStat software.
Ethical considerations

The study was reviewed and approved by the Ethics Committee of the Virgen de la Arrixaca Hospital. Written informed consent was obtained from all patients enrolled in the study.

Results

PCR as a diagnostic tool

To assess parasitological status before benznidazole treatment, PCR was performed on the 181 blood samples from the *T. cruzi* seropositive chronic patients and was positive in 123 samples (68%). PCR was found to be a sensitive parasitological test.

PCR positives were significantly more frequent in young patients than in adult and senior patients (*P* = 0.0007) (100% in young patients, 72.1% in adult patients and 48.9% in senior patients). Moreover, PCR positives were significantly more frequent in short-term (78.5%) than in long-term (59.8%) immigrants (*P* = 0.0076).

On the other hand, no differences in PCR sensitivity were observed between samples of patients with chronic symptomatic (64.6%) and indeterminate (69.8%) Chagas’ disease (*P* = 0.4710). Table 1 shows the results of PCR related to groups of the study population.

PCR as a post-treatment parasitological follow-up tool

Ninety days post-treatment, 96 PCRs were performed on 123 patients whose PCR was initially positive. All proved negative (100%). Furthermore, 26 of the remaining 58 patients with a negative PCR before treatment were studied at the same time post-treatment and all remained negative. The results are summarized in Figure 2.

Full serology and PCR data for the 420 day follow-up period are available for 58 patients. The results show that serology still remained positive and no significant change in serum titres was observed 420 days after treatment (Table 2). A total of 42 patients had a positive PCR result before treatment, which became negative at 90 days in 38 of the patients, remaining negative until the end of the follow-up period (Figure 2). In contrast, the PCR became negative at 90 days, but had shifted to positive by 420 days, in 4 of the 42 chronic patients correctly

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**Table 1.** PCR results before treatment according to groups of the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCR positive, n/total (%)</th>
<th>PCR negative, n/total (%)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>young, 2–19 years</td>
<td>12/12 (100)</td>
<td>0/12 (0)</td>
<td>0.0007</td>
</tr>
<tr>
<td>adult, 20–39 years</td>
<td>88/122 (72.1)</td>
<td>34/122 (27.9)</td>
<td></td>
</tr>
<tr>
<td>senior, ≥40 years</td>
<td>23/47 (48.9)</td>
<td>24/47 (51.1)</td>
<td></td>
</tr>
<tr>
<td>Years of residence in Spain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>short-term, &lt;3 years</td>
<td>62/79 (78.5)</td>
<td>17/79 (21.5)</td>
<td>0.0076</td>
</tr>
<tr>
<td>long-term, ≥3 years</td>
<td>61/102 (59.8)</td>
<td>41/102 (40.2)</td>
<td></td>
</tr>
<tr>
<td>Symptomatology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>asymptomatic</td>
<td>81/116 (69.8)</td>
<td>35/116 (30.2)</td>
<td>0.4710</td>
</tr>
<tr>
<td>symptomatic</td>
<td>42/65 (64.6)</td>
<td>23/65 (35.4)</td>
<td></td>
</tr>
</tbody>
</table>

*aTotal patients = 181.*
observing treatment and in 1 at 150 days who did not complete the treatment. A total of 15 patients had a negative PCR result before treatment and remained negative throughout the follow-up. Only in one patient with a negative PCR before and at the end of the follow-up did the PCR shift to positive at 150 days.

No significant correlation was found between the patients’ ages and positive PCR results at the end of follow-up ($P = 0.3261$).

**Discussion**

This study is the first of its kind to assess the usefulness of PCR amplification of kinetoplast minicircle DNA sequences as a post-treatment parasitological follow-up tool in chronic Chagas’ disease patients in a European country, where the possibility of re-infection can be discarded.

The sensitivity of PCR compared with serology methods for the diagnosis of Chagas’ disease has been found to be between 59% and 100%, depending on several factors, such as the geographical area and the age of the subjects. In this study PCR was performed on 181 samples of chronic patients infected by $T. cruzi$, with a sensitivity of 68% (123). The patients were mainly from Bolivia (97.2% of the study population), for whom PCR shows high sensitivity. On the other hand, taking into account the age range of our patients, it is unsurprising that we found different degrees of PCR sensitivity for the three age groups. PCR diagnostic sensitivity compared with serology was 100% in young patients (2–19 years), 72.1% in adult patients (20–39 years) and 48.9% in senior patients ($\geq 40$ years). At the beginning of the study, a positive PCR result was more frequent among newly arrived individuals, probably because of the shorter time elapsed between infection and testing.

Whether or not PCR-based detection of $T. cruzi$ amplifies the DNA of intact parasites or lysed parasites from infected cells is not clear. Contrary to the results reported in other studies, we detected an early PCR-negative conversion in all patients 90 days post-treatment ($n = 96$). Considering that the suppressive activity against parasitaemia is almost immediate after the initiation of treatment, our results highlight the fact that PCR is a sensitive and specific tool for the early detection of the parasite’s susceptibility to benznidazole, allowing early therapy modification in cases of resistance or reactivation of chagasic infection.

Specific treatment has been recommended for the acute phase of $T. cruzi$ infection and, recently, for all patients with Chagas’ disease. Nevertheless, cure assessment in chronic
infection is controversial, mainly because of the lack of reliable tests to ensure parasite elimination. In accordance with the findings of previous studies, no significant negative seroconversion was observed 420 days post-treatment in patients with chronic infection.\textsuperscript{18,20} Seroconversion occurs several years after treatment, requiring long-term follow-up. The high sensitivity and specificity of the PCR-based diagnosis of \textit{T. cruzi} infection found in our study suggest that it is a suitable tool for the follow-up of benznidazole-treated patients with chronic Chagas' disease.

The PCR becomes negative in almost all the patients, showing better than the serology the impact and maybe the benefit of the treatment. A negative PCR result after treatment does not guarantee the cure of the patients in the absence of other markers. However, PCR allows treatment failure to be ratified in patients whose PCR shifts to positive in a short-term follow-up.

The effectiveness of benznidazole in eradicating \textit{T. cruzi} during the chronic phase of the disease is also controversial.\textsuperscript{21} However, very few studies have been conducted to evaluate antiparasitic treatment using PCR in chronic cases, and the few investigations that have been carried out were done in Latin America, some of them in highly endemic areas, in which active transmission occurs continuously.\textsuperscript{22}

According to our results 100% of the treated patients had a negative PCR result 90 days post-treatment, reflecting the effectiveness of benznidazole at clearing parasites. This high rate of therapeutic effectiveness assessed by PCR in our study is higher than the rates reported in other studies. Nevertheless, it should be remembered that this is the first study performed in a vector-free area where the absence of trinominal vectors rules out the possibility of re-infection during follow-up. Only 6.9% of the treated patients remained PCR positive after chemotherapy. However, this is a short-term study and, taking into account the waves of parasitaemia that occur during the long course of Chagas' disease, among the treated chronic patients presenting a negative PCR 420 days post-treatment, some would be expected to shift to a positive PCR during long-term follow-up.

No significance was found between the patients' ages and a positive PCR result at the end of follow-up, although all treated patients with a positive PCR at this time were in the adult and senior groups.

This well-characterized group of patients with Chagas' disease is presently being followed up to confirm treatment efficacy and the correlation between a PCR-negative test and a decrease in antibody titres in the patients treated with benznidazole.

\section*{Acknowledgements}

We thank Manuel Carlos Lopez and María Carmen Thomas for their helpful comments on the study, and María Carmen Martínez, Carmen Marquez, Fueonsanta Franco and Carmen Zamora for their technical assistance.

\section*{Funding}

This study was supported by the Spanish Ministry of Science and Innovation and the Instituto de Salud Carlos III within the Network of Tropical Diseases Research (RICET RD06/0021/1007) and the Project of Research in Health (PS09/01956).

\section*{Transparency declarations}

None to declare.

\section*{References}