ELSEVIER

Diagnostic Microbiology and Infectious Disease
48 (2004) 253–257
Parasitology

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

www.elsevier.com/locate/diagmicrobio

Use of polymerase chain reaction (PCR) and hybridization assays to detect *Trypanosoma cruzi* in chronic chagasic patients treated with itraconazole or allopurinol

Inés Zulantay[a,*], Patricia Honores[a], Aldo Solari[a], Werner Apt[a], Sylvia Ortiz[a], Antonio Osuna[b], Antonio Rojas[a], Bernardo López[a], Gittith Sánchez[a]

[a]*Cellular and Molecular Biology Program, Biomedical Sciences Institute, Faculty of Medicine, University of Chile, Santiago, Chile*
[b]*Institute of Biotechnology, Department of Parasitology, Faculty of Science, University of Granada, Granada, Spain*

Received 15 April 2003; accepted 7 October 2003

Abstract

The presence of *Trypanosoma cruzi* in chronic chagasic patients with negative xenodiagnosis (XD) after 6 years following completion of therapy with either itraconazole or allopurinol was assessed by polymerase chain reaction (PCR) and hybridization assays. A 330-bp DNA fragment amplified from the hypervariable regions of *T. cruzi* kinetoplastid minicircles was hybridized with total ^{32}P-labeled kinetoplast DNA as probes. PCR alone enabled the identification of *T. cruzi* nucleotide sequences in 40% of the patients treated with itraconazole and in 60% of patients treated with allopurinol. PCR used in combination with hybridization detected parasite DNA in 60% and 53% of XD negative individuals treated with itraconazole or allopurinol, respectively. These results show that PCR and hybridization are more sensitive than conventional parasitological techniques in diagnosing patients that have undergone chemotherapy with itraconazole or allopurinol.

Keywords: Chronic Chagas disease; *Trypanosoma cruzi*; Polymerase chain reaction (PCR); Hybridization assays; Itraconazole; Allopurinol

1. Introduction

PCR has been successfully used to amplify variable regions of *T. cruzi* kinetoplast DNA, which is abundant and can be easily prepared from the parasite (Britto et al.,1993; Junqueira et al., 1996). Oligonucleotides targeting conserved regions of the *T. cruzi* minicircles have been designed to amplify a 330-bp DNA fragment from a variable region of the parasite minicircles (Sturn et al., 1989). Studies with sera taken from acute and chronic chagasic individuals that did not receive antiparasitic therapy showed PCR amplification of *T. cruzi* DNA in all the samples analyzed (Russomando et al., 1992). In addition, Junqueira et al. (1996), determined 59.4% of sensitivity with PCR in blood samples of serologically positive chronic chagasic individuals in comparison with 35.6% and 25.7% obtained for xenodiagnosis (XD) and hemoculture, respectively. Furthermore, PCR has been reported to provide efficient 90% detection with *T. cruzi* with seropositive results for Chagas disease (Britto et al., 1995). Recently, Marcon et al. (2002), found that 43 of 50 patients previously serodiagnosed as chagasic were positive using nested PCR (N-PCR). These authors suggested that N-PCR may be a complementary tool to for parasite detection in patients with doubtful serologic results. Evaluation of the efficacy of chronic Chagas disease treatment is hampered by the lack of reliable criteria of cure as well as the rather poor sensitivity of conventional parasitological practices such as XD (Wincker et al., 1994). For these reasons, PCR has become a useful tool also to detect the presence of *T. cruzi* in chronic chagasic patients undergoing chemotherapy. Britto et al. (2001) compared PCR with xenodiagnosis in chronic chagasic patients after chemotherapy with nifurtimox or benznidazol. They observed that PCR was negative in 65% of the patients while 83% led to xenodiagnosis negative results. Solari et al. (2001b) demonstrated that *T. cruzi* could be detected by PCR in all children of an infected but untreated population and in 69% of an adult population that also remained untreated. The XD positivity determined for the same groups was 79% and

* Corresponding author. Tel.: +56-2-678-6122; fax: +56-2-735-6579.
E-mail address: izulanta@machi.med.uchile.cl (I. Zulantay).

0732-8893/04/$ – see front matter
doi:10.1016/j.diagmicrobio.2003.10.011

Table 1
Detection of *Trypanosoma cruzi* kDNA by PCR and hybridization with total kinetoplast DNA probes of ^{32}P-labeled parasites in peripheral blood of 52 treated chronic chagasic patients classified according to xenodiagnosis (XD) and drug received

Group	*n*	Drug	XD Post-Therapy	PCR + *n* (%)	Hybridization + *n* (%)
I	15	itraconazole	Negative	6 (40)	9 (60)
II	11	itraconazole	Positive	9 (81.8)	9 (81.8)
III	15	allopurinol	Negative	9 (60)	8 (53)
IV	11	allopurinol	Positive	9 (81.8)	9 (81.8)

21%, respectively. While all patients converted to negative XD within 3-6 months after nifurtimox therapy, PCR remained positive for a longer period of time. The present work reports the usefulness of PCR and hybridization to monitor parasite clearance in chronic chagasic patients who had completed a therapeutic course with itraconazole or allopurinol 6 years earlier and whose XD results were negative.

2. Material and methods

2.1. Patients

A total of 52 chronic chagasic individuals (mean age, 41 years old) from a highly endemic region of Chile (Regions IV and V) were treated with itraconazole or allopurinol. All patients were classified according to XD tests performed parallel to taking of blood samples for PCR at 6 years after treatment. As a means to increase the sensitivity of parasitemia detection, the amplified PCR products were hybridized with a universal kDNA probe (Solari, 2001a). Patients were classified according to the drug used and XD, as shown in Table 1. Patients came from regions that are under epidemiologic surveillance by the Vector Control Program of the Ministry of Health and the Initiative of South Cone (World Health Organization, 2000).

2.2. Chemotherapy protocol

Chagasic patients were selected from a randomized, double-blind study to evaluate the efficacy and tolerance of itraconazole (n = 26; 6 mg/kg/day in 100 mg capsules; Janssen Laboratories, Beerse, Belgium) administered as directly observed therapy (DOT) divided in two doses per day over a 120-day period, or allopurinol (n = 26; 300 mg tablets; Silesia Laboratories, Chile) at a daily dose of 8.5 mg/kg for60 days. The study was approved by the Ethics Committee of the Faculty of Medicine of the University of Chile, and informed consent was obtained from each patient (Apt, 2003).

2.3. Xenodiagnosis

The XD test was carried out simultaneously with the blood sampling for PCR using two cylindrical wooden vials (seven third-instar of *Triatoma infestans* each). After feeding, a microscopic examination of insect faeces was performed at 30, 60, or 90 days of incubation at 27°C, when the previous examination was negative. All of the microscopic fields in a 2.2 cm area were examined twice (Schenone, 1999).

2.4. Polymerase chain reaction (PCR)

Two milliliters of blood was mixed with the same volume of a 6 M guanidine hydrochloride/200 mM EDTA solution, boiled for 15 min, and stored at 4°C. DNA was extracted from 500 μl of the mixture using the phenol-chloroform protocol and precipitated with ethanol as described by Wincker et al. (1994). DNA was resuspended in 50 μl of water, chromatographed in a P10 filtration microcolumn, and stored at −20°C. In parallel, an external control of PCR was performed by the Research Group of Molecular Parasitology, Institute of Biotechnology, University of Granada, Spain, using essentially the same protocol with some differences, such as the use of Proteinase K, isoamilic alcohol in the DNA extraction and different origin of primers as well as all reagents used. Reactions of PCR were carried out as described by Solari et al. (2001a). Briefly, 5 μl of template DNA was amplified in a final volume of 50 μl containing 0.25 mM of each of dATP, dCTP, dGTP, and dTTP, and 200 ng of forward and reverse *T. cruzi*-specific primers 121 (5′ AAATAATGTACGG(T/G)GAGATGCATGA-3′) and 122 (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′), 2.5 U of Taq polymerase, 5 μl of 10× Taq DNA polymerase buffer, and 6.7 mM $MgCl_2$. Mineral oil (80 μl) was added to avoid reaction mixture evaporation. The PCR condition was performed to ensure that all fragment were completely synthesized (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; a final incubation at 72°C for 10 min in a thermocycler). PCR internal control for amplification of the human β-globin gene sequences was as described by Saiki et al. (1985). Routinely, on a set of five lysates from patients to be diagnosed, one negative blood sample from a noninfected person was included. A single amplification of a DNA preparation originating from a confirmed chagasic patient was included as positive control, and a tube without DNA was included as a negative control. A 330-pb product represented a positive assay. PCR assays were performed in duplicate. PCR products were fractionated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under an ultraviolet light.

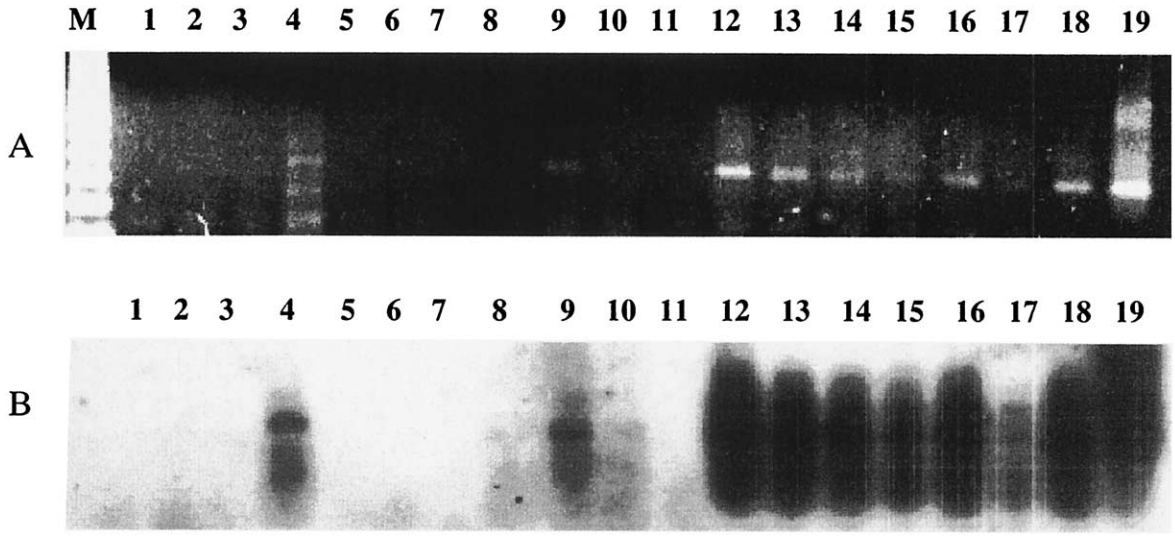

Fig. 1. A, Electrophoretic pattern of minicircle region 330 bp in size of kDNA-PCR amplified with primers 121 and 122 from different human subjects and stained with ethidium bromide. B, DNA of A transferred to a nylon membrane, hybridized with ^{32}P-labeled with total kDNA as a probe. Lanes 2–18 show 17 patients with positive or negative PCR-based diagnosis. Lane 19 is a sample from a positive control. Lane M is a 100-bp ladder as a molecular size marker.

2.5. Southern blot analysis

Amplified DNA was then transferred to a nylon membrane, denatured, and cross-linked by UV irradiation. The membranes were prehybridizated in 6× SSC and 1 mM EDTA, pH 7.4, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 20 μg/ml denatured salmon-sperm DNA at 65°C, and hybridized overnight in the same solution with the labeled probe. Washing was carried out in 0.1× SSC. Hybridization were carried out using total kDNA from *T. cruzi* as a universal probe and labeled by the random priming method with (α^{32}P)dCTP (Solari et al.,1991). The membranes were autoradiographed for from 2 to 24 h. (Solari et al., 2001a). All tests were performed in duplicate.

2.6. Statistics

All results were analyzed using the χ^2 statistical test, and p values <0.05 were considered to be significant.

3. Results

T. cruzi DNA detection was considered positive when both a band of 330 pb by PCR and positive hybridization were demonstrated (Fig. 1), or, in a few cases, when PCR was negative and hybridization positive. These criteria were applied on the basis of the description of the higher sensitivity and specificity of hybridization test that uses radiolabeled probes instead of ethidium bromide stain (Breniere et al., 1998). As can be seen in Table 1, in 6 of 15 cases with negative XD after treatment with itraconazole (Group I), *T. cruzi* DNA was assessed by PCR and were also confirmed by the hybridization test. Three of the nine remaining cases that were negative by PCR proved positive in the hybridization test. These figures mean that 40% of the samples with negative XD proved positive by PCR, and this percentage increased up to 60% when the hybridization test was applied. These results were statistically significant when compared with those obtained by XD ($p < 0.0001$). In Group II, composed of individuals with positive XD after treatment with itraconazole, PCR were positive in 9 of 11 cases and all 9 were confirmed by hybridization; therefore, no statistically significant differences were found when a comparison was made with the results obtained by XD. In the Group III (negative XD after treatment with allopurinol), 9 of 15 cases were positive by PCR, 8 of these also being positive by hybridization. When the results by PCR and hybridization tests were compared with those results of XD, statistical significance was found ($p < 0.0001$). In the Group IV, positive XD after treatment with allopurinol, 9 of 11 cases were positive by PCR and hybridization test, with no statistically significant differences with respect to the results of XD. In the four cases belonging to the control Groups II and IV, with no detectable *T. cruzi* kDNA by PCR or hybridization, PCR was performed using specific primers for human β-globin gene (Britto et al.,1995), with positive results in all cases; thus, the presence of inhibitors in those samples was discarded. In 40 of 52 samples (70%), PCRs were also performed blind, without knowing the previous results in the Biotechnology Center of University of Granada, Spain with 90% of agreement. The four cases of disagreement belonged to Group I ($n = 3$) and Group III ($n = 1$). In those cases, hybridization tests were considered the definitive criteria (3 negative and 1 positive).

4. Discussion

The chronic phase of Chagas disease is characterized by subpatent parasitemia and scarce tissue parasitism (Gomes at al; 1999). In this phase, the usefulness of parasitological techniques such as XD applied as evaluation tools of chemotherapeutic efficacy is limited, since the sensitivity of the techniques depends on varying density of parasites in blood (Britto et al., 1995; Junqueira et al., 1996). For this reason, negative results do not necessarily indicate a lack of parasitemia or a parasitological cure after treatment. The recent use of PCR to detect *T. cruzi* in blood samples of chagasic patients has opened new possibilities in the diagnosis and evaluation of chemotherapeutic treatment. The present study, performed 6 years after therapy allows the detection of the presence of kDNA from *T. cruzi* in 40% of the patients treated with itraconazole, whose XD performed at same time of the blood sample uptake for PCR were negative. The PCR results were confirmed by hybridization tests in 88.5% and 92.2% of the patients treated with itraconazole and allopurinol, respectively. This high concordance between both methods of *T. cruzi* DNA detection has also been described by other authors (Breniere et al., 1998; Gomes et al., 1999). In three samples from patients treated with itraconazole, with negative PCR, it was possible to detect the 330-pb band when the universal probe was labeled with ^{32}P (11.5%), demonstrating that hybridization is more sensitive than PCR, as has been reported elsewhere (Breniere et al., 1998). In one case treated with allopurinol, the reverse situation occurred, since hybridization was negative although PCR was positive. This fact could be due to an inadequate transference of the amplified DNA to the nylon membrane or an insufficient quantity of DNA for hybridization. This case was considered negative by the criterion of positivity accepted in the present study. With respect to Groups II and IV, each comprised of 11 patients with positive XD after therapy, in 9 cases of each group, it was possible to detect *T. cruzi* kDNA. The two negative cases belonging to each group could be explained by the absence of parasites in the blood sample collected, thus suggesting that the sample volume would be an important factor to be considered in chronic patients with low levels of parasitemia. Another explanation would be the presence of PCR inhibitors. This possibility was ruled out because under the same conditions, amplification assays performed using two specific primers for human β-globin gene gave positive amplification. In this regard, Junqueira et al. (1996) reported four cases whose XD were positive and PCR negative, suggesting the presence of PCR inhibitors as an explanation of this fact. On the other hand, they also described the evaluation of two cases whose PCR were negative and showed β-globin gene amplification, concluding that those results were not due to inhibition of the reaction. This situation was also described by Britto et al. (1995). The results found in the present study show that molecular biology tools are more sensitive for demonstrating parasite persistence in chemotherapeutic evaluation.

Acknowledgments

This work was supported by DI-SAL 03/6-2 Fondecyt 1040731. Vicerrectoría de Investigación y Desarrollo (DI) Universidad de Chile, IAEA RLA 6/026 and Instituto de Biotecnología. Universidad de Granada, España.

References

Apt, W., Arribada, A., Zulantay, I., Sánchez, G., Vargas, S. L., & Rodríguez, J. (2003). Regression and prevention of electrocardiographic abnormalities in chronic chagasic patients treated with itraconazole or allopurinol: a nine years follow-up study. *Ann Trop Med Parasitol 97 (1)*, 23–29.

Breniere, S., Bosseno, M. F., Melleria, J., Bastrenta, B., Yaesik, N., Noireau, F., Alcazar, J. L., Barnabé, C., Wincker, P., & Tibayrenc, M. (1998). Different behavior of two *Trypanosoma cruzi* major clones: transmission and circulation in young bolivian patients. *Exp Parasitol 89*, 285–295.

Britto, C., Cardoso, M. A., Wincker, P., & Morel, C. M. (1993). A simple protocol for the physical cleavage of *Trypanosoma cruzi* kinetoplast DNA present in blood samples and its use in polymerase chain reaction (PCR)-based diagnosis of chronic Chagas'disease. *Mem Inst Oswaldo Cruz 88*, 171–172.

Britto, C., Cardoso, M. A., Ravel, C., Santoro, A., Borges-Pereira, J., Coura, J. R., Morel, C. M., & Wincker, P. (1995). *Trypanosoma cruzi*: Parasite detection and strain discrimination in chronic chagasic patients from northeastern Brazil using PCR amplification of kinetoplast DNA and nonradioactive hybridization. *Exp Parasitol 81*, 462–471.

Britto, C., Silveira, C., Cardoso, M. A., Marques, P., Luquetti, A., Macedo, V., & Fernandes, O. (2001). Parasite persistence in treated chagasic patients revealed by xenodiagnosis and polymerase chain reaction. *Mem Inst Oswaldo Cruz 96 (6)*, 823–826.

Gomes, M., Galvao, L., Macedo, A., Pena, S., & Chiari, E. (1999). Chagas'disease diagnosis: comparative analysis of parasitologic, molecular and serologic methods. *Am J Trop Med Hyg 60 (2)*, 205–210.

Junqueira, A. C., Chiari, E., & Wincker, P. (1996). Comparison of the polymerase chain reaction with two classical parasitological methods for the diagnosis of Chagas disease in a endemic region of northeastern Brazil. *Trans Roy Soc Trop Med Hyg 90 (2)*, 129–132.

Marcon, G. E., Andrade, P. D., De Albuquerque, D. M., Wanderley, S., De Almeyda, E. A., Guariento, M. E., & Costa, S. C. (2002). Use of a nested polymerase chain reaction (N-PCR) to detect *Trypanosoma cruzi* in blood samples from chronic chagasic patients and patients with doubtful serologies. *Diagn Microbiol Infect Dis 43 (1)*, 39–43.

Russomando, G., Figueredo, G., Almirón, M., Sakamoto, M., & Morita, K. (1992). Polymerase chain reaction-based detection of *Trypanosoma cruzi* DNA in serum. *J Clin Microbiol 30*, 2864–2868.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Aeneihm, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science 230*, 1350–1354.

Schenone, H. (1999). Xenodiagnosis. *Mem Inst Oswaldo Cruz 94 (I)*, 289–294.

Solari, A., Campillay, R., Ortiz, S., & Wallace, A. (2001a). Identification of *Trypanosoma cruzi* genotypes circulating in chilean chagasic patients. *Exp Parasitol 97*, 226–233.

Solari, A., Ortiz, S., Soto, A., Arancibia, C., Campillay, R., Contreras, M., Salinas, P., Rojas, A., & Schenone, H. (2001b). Treatment of *Trypanosoma cruzi*-infected children with nifurtimox: a 3 year follow-up by PCR. *J Antimicrob Chemother 48 (4)*, 515–519.

Solari, A., Venegas, J., González, E., & Vásquez, C. (1991). Detection and classification of *Trypanosoma cruzi* by DNA hybridization with non radioactive probes. *J Protozool 38*, 559–565.

Sturn, N. R., Degrave, W., Morel, C., & Simpson, I. (1989). Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences; use in diagnosis of Chagas disease. *Mol Biochem Parasitol 33*, 205–214.

Wincker, P., Britto, C., Pereira, J. B., Cardoso, M. A., Oeleman, O., & Morel, C. M. (1994). Use a simplified polymerase chain reaction procedure to detect *Trypanosoma cruzi* in blood samples from chronic chagasic patients in a rural endemic area. *Am J Trop Med Hyg 51*, 771–777.

World Health Organization (2000). Chagas'disease interrupted in Chile. *TDR News 61*, 10.