ARTIGO ORIGINAL

USE OF THE POLYMERASE CHAIN REACTION (PCR) FOR EARLY EVALUATION OF ETIOLOGICAL TREATMENT IN YOUNG ADULTS, CHRONICALLY INFECTED WITH *Trypanosoma cruzi*

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ABSTRACT

Fourteen patients (age limits: 18-30) chronically infected with *Trypanosoma cruzi* and presenting positive blood PCR reactions, were submitted to etiological treatment with benznidazole, in Salta, Argentina. A control group of five patients of the same age and positive by PCR did not receive treatment. Post-treatment follow-up was performed with PCR and conventional serology. PCR became negative in 12/14 (85.7%) of the treated patients after six months follow up, compared to 1/5 (20%) in the control group ($p = 0.01$). All patients remained serologically positive after treatment. The reduction of PCR signals of infection after treatment may become an early evidence of cure in chronically infected young adult patients.

KEYWORDS: Chagas disease. Etiological treatment. PCR.

INTRODUCTION

Trypanosoma cruzi infects around 18 million persons in an endemic area from northern Mexico to southern Argentina (15). The population at risk of infection is estimated at 40 millions (15), and Chagas disease is one of the major causes of morbi-mortality in the region. Additionally, this disease has extended, due to migrations, to countries such as Spain (6) and the USA (7).

The etiological treatment of chronically infected patients has been recommended by the World Health Organization (10). Brazil (8) and Argentina (9) treatment guidelines also recommend treatment for such patients, stating that the

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decision should be taken by each patient and his/her physician. Conventional serologic (Enzyme Linked Immunosorbent Assay - ELISA, Indirect Hemagglutination - IHA) and parasitologic (Hemoculture, Xenodiagnosis) methods are recommended for the control of therapeutic efficacy. However, in the chronic phase, conventional reactions only become negative years after treatment (1, 10), and conventional parasitologic methods have low sensitivity (4). The Polymerase Chain Reaction (PCR), detecting *Trypanosoma cruzi* kDNA (13), showed higher diagnostic sensitivity than conventional methods in chronic Chagas disease patients (14).

In this work, we explored the usefulness of PCR as a tool for early evaluation of the efficacy of etiologic treatment in young adults infected by *T. cruzi.* Treatment was also controlled with conventional serology (IHA, ELISA).

MATERIALS AND METHODS

Subjects

Ninety-one patients were interviewed, age ranged from 18 to 30 years old, and they had two positive serological tests (ELISA and IHA). The PCR test was applied to all of them. The reaction was positive in 36/91 (65.5%). Treatment was initiated in 18 patients and 14 completed the whole treatment course. The control group was composed of five patients who rejected treatment or received it after this study. Out of 19 patients thirteen were women. All lived in Salta city, Argentina, an area with no vectorial transmission, and signed an informed consent. Exclusion criteria were: previous treatment for *T. cruzi*, pregnancy, liver or kidney disease, low leukocyte or platelet counts, alcoholism, severe pathology associated to Chagas disease and breast-feeding. The project was approved by the Bioethics Commission of the Faculty of Medical Sciences of the University of Rosario, Argentina, and of the Faculty of Health Sciences of the University of Salta, Argentina.

Treatment

Two groups were studied: the treated group (TG), included 14 patients (age range 23.6 ± 3.5 years), which received benznidazole at 5 mg/kg/day for 60 days, by oral route. The control group (CG), included 5 patients (age range 26.6 ± 4.9 years), which did not receive treatment. The follow-up was of 6 months post-treatment for TG and from 2 weeks to 6 months for CG.

Serology

After separating the serum, IHA and ELISA tests were done, using Wiener Laboratory kits (Argentina) and following the recommended procedures and cut-off points (1/16 dilution for IHA and 0.22 absorbance for ELISA).

PCR

The procedure used by Britto et al. (3) with minor modifications was used. Blood (5 mL) was diluted 1:1 in 6 M-Guanidine EDTA 0.2 M buffer. The sample was heated at 100º C 10 minutes. DNA was extracted in minipreps using 100 µl sample aliquots, using phenol-chlorophorm-isoamilic alcohol and sodium acetate-ethanol precipitation. Amplification mixtures included primers 121 (5'-AAA TAA TGT ACG GGT GAG ATG CAT GA-3') and 122 (5'-GGT TCG ATT GGG GTT GGT GTA ATA TA-3'). Amplification was carried out in a thermal cycler (M. J. Research, Watertown, Masssachusetts, USA) with the following cycle sequence: cycle 1(x2) 1 min at 98º C and 2 min at 64º C; cycle 2 (x33): 1 min at 94 ºC and 2 min at 64º C; cycle 3 (x1): 1 min at 72º C and 2 min at 25º C. Amplified products were electrophoresed in 0.2 % agarose minigels, stained with ethidium bromide and revealed with ultraviolet light. Positive samples displayed a 330 bp band. Because cross-contamination or PCR artifacts are a constant risk, a maximum of 5 samples was processed, together with a negative and a positive control, per extraction session. PCR mixtures and DNA extraction were performed in separate chambers. All reagents were prepared in aliquots, using exclusive pipettes and filter tips. Amplicons were electrophoresed in a separate room.

Statistical analysis

The significance of differences between proportions was calculated with the Fisher's exact test.

RESULTS

The main outcomes of ELISA, IHA, and PCR for each patient are shown in Tables 1 and 2. PCR became negative in 12/14 (85.7%) TG patients at month 6 post-treatment, and in 1/5 (20%) CG patients ($p = 0.01$). All patients remained serologically positive after treatment (TG), or in the follow-up of the CG.

Seven of 14 (50%) patients in the TG presented adverse effects of benznidazole. These were: allergic dermatitis (4/7; 57.2%), peripheral polyneuritis (2/7; 28.6%), and asthenia (1/7; 14.3%). Nevertheless, treatment could be completed in all patients, by either diminishing temporarily the dose or by symptomatic medication. In two cases, adverse effects disappeared spontaneously after the first days of treatment.

Table 1. Results of serological tests and PCR before/after treatment with benznidazole of young adults, infected with *Trypanosoma cruzi*, Salta, Argentina

Pt	Before treatment			After treatment		
	IHA	ELISA	PCR	IHA	ELISA	PCR
1	1/1024	pos	pos	1/1024	pos	neg
2	1/32	pos	pos	1/16	pos	neg
3	1/256	pos	pos	ND	ND	neg
4	1/512	pos	pos	1/128	pos	neg
5	1/64	pos	pos	1/256	pos	pos
6	1/256	pos	pos	1/32	pos	neg
7	1/512	pos	pos	1/256	pos	neg
8	1/1024	pos	pos	1/512	pos	neg
9	1/1024	pos	pos	1/512	pos	neg
10	1/1024	pos	pos	1/512	pos	pos
11	1/1024	pos	pos	1/1024	pos	neg
12	1/64	pos	pos	1/512	pos	neg
13	1/1024	pos	pos	1/512	pos	neg
14	1/32	pos	pos	1/64	pos	neg

Pt: patients; IHA: Indirect Hemagglutination; ELISA: Enzime Linked Immunosorbent Assay; pos: positive; neg: negative; ND: not done.

Table 2. Results of serological tests and PCR of a group of non-treated, *Trypanosoma cruzi* infected, young adults from Salta, Argentina

Pt	Beginning			Follow-up		
	IHA	ELISA	PCR	IHA	ELISA	PCR
A	1/64	pos	pos	ND	ND	pos
B	1/128	pos	pos	1/256	pos	pos
C	1/64	pos	pos	ND	ND	neg
D	1/16	pos	pos	ND	ND	pos
E	1/128	pos	pos	ND	ND	pos

Pt: patients; pos: positive; neg: negative; ND: not done.

DISCUSSION

The criterion of cure for Chagas disease is still the negativization of conventional serologic and parasitologic tests (5), even though there is not much experience using this criterion in chronically infected adults. During this stage of the disease, conventional parasitologic tests display low sensitivity and serological reactions become negative very late, even in cases of successful treatment (1, 10). PCR could overcome both difficulties: it could provide results on the efficacy of treatment with higher sensitivity than conventional parasitological tests, and earlier in time than serological tests.

In this work, a significant negativization of PCR was demonstrated in the TG. Although these results are promising for monitoring treatment, the relatively low sensitivity of PCR should be considered. 65.5% of chronically infected patients were positive by PCR. Similar results were observed by other authors (11). A negative result after treatment therefore does not indicate cure, although it could provide a supporting evidence of cure.

Only two patients presented a positive PCR after treatment. The possibilities of contamination and lack of treatment compliance were reasonably discarded. Therefore, these results could indicate failure to therapy. This fact has been shown in other studies (2), and could be due to infection with resistant parasite strains (12).

An important proportion of adverse effects of benznidazole was observed. However, most of them were mild or could be resolved with simple clinical indications.

To evaluate the effectiveness of PCR as method for assessing therapy in chronically infected adults, its sensitivity should be increased and patients should be followed-up for longer periods.

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RESUMO

Uso da Reação em Cadeia da Polimerase para a avaliação precoce do tratamento etiológico em adultos jovens infectados crônicos por *Trypanosoma cruzi*

Foi realizado o tratamento etiológico com benznidazol, no Estado de Salta, Argentina, em 14 pacientes infetados crônicos por *Trypanosoma cruzi*, de 18 a 30 anos de idade, com Reação em Cadeia da Polimerase (PCR) positiva. Um grupo controle de cinco pacientes, da mesma idade, também com PCR positiva, não recebeu tratamento. O seguimento após tratamento foi realizado com PCR e sorología convencional. Após 6 meses de tratamento foi observada negativização da PCR de 14/16 (85,7%) nos pacientes tratados *versus* 20% no grupo controle (p = 0,001). A sorologia foi positiva em todos os pacientes depois do tratamento. Os resultados da PCR pós-tratamento, podem ser um indício de cura no tratamento de infectados chagásicos crônicos, adultos jovens.

DESCRITORES: Doença de Chagas. Tratamento etiologico. Reação em Cadeia da Polimerase.

REFERENCES

1. Andrade SG. Tratamiento específico experimental da Doença de Chagas. *Rev Patol Trop 29 (supl):* 179-189, 2000.
2. Braga MS, Lauria-Pires L, Arganaraz ER, Nascimento RJ, Teixeira AR. Persistent Infections in Chronic Chagas' disease patients treated with anti-*Trypanosoma cruzi* nitroderivates. *Rev Inst Med Trop São Paulo 42:* 157-161, 2000.
3. Britto C, Cardoso MAB, Wincker P, Morel CM. A simple protocol for the physical cleavage of *Trypanosoma cruzi* kinetoplast DNA present in blood samples and it use in polymerase chain reaction (PCR) – based diagnosis of chronic Chagas disease (Technical note). *Mem Inst Oswaldo Cruz 88:* 171-172, 1993.
4. Bronfen E, Rocha FSA, Machado GBN, Perillo MM. Isolamento de amostras do *Trypanosoma cruzi* por Xenodianóstico e hemocultura de pacientes na fase crônica da doença de Chagas. *Mem Inst Oswaldo Cruz 84:* 237-240, 1989.
5. Cançado R, Brener Z. Terapêutica. In Z Brener, Z Andrade (eds) *Trypanosoma cruzi e Doença de Chagas*, Guanabara Koogan, Rio de Janeiro, 1979.
6. Florian Sanz F, Gomez Navarro C, Castrillo Garcia N, Pedrote Martinez A, Lage Galle E. Chagasic cardiomyopathy in Spain: a diagnosis to bear in mind. *An Med Interna 22:* 538-540, 2005.
7. Kirchoff LV. American trypanosomiasis (Chagas' disease), a tropical disease now in the United States. *N Engl J Med 329:* 639-644, 1993.
8. Ministério da Saúde. *Tratamento Etiológico da Doença de Chagas*. 2nd ed., Fundação Nacional de Saúde, Brasilia, 1997.
9. Normas para la atención al paciente infectado con *Trypanosoma cruzi* (Enfermedad de Chagas). Ministerio de Salud y Medio Ambiente de la República Argentina, 2005.
10. OPS/HCP/HCT/140/99. Tratamiento etiológico de la enfermedad de Chagas: conclusiones de reunión de especialistas. *Rev Patol Trop 28:* 247-279, 1999.
11. Portela-Lindoso AA, Shikanai-Yasuda MA. Chronic Chagas' disease: from xenodiagnosis and hemoculture to polymerase chain reaction. *Rev Saude Publica 37:* 107-115, 2003.
12. Stoppani AOM. Quimioterapia de la enfermedad de Chagas. *Medicina (Buenos Aires) 59 (Supl II):* 147-65, 1999.
13. Sturm NR, Degrave W, Morel C, Simpson L. 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, 1989.
14. Wincker P, Britto C, Borges Pereira J, Cardoso MA, Oeleman W, Morel C. Use of 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, 1994.
15. World Health Organization. Control of Chagas Disease. *Tech Rep Ser 905*: 1-109, 2002.