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SHORT COMMUNICATION

Parasite Persistence in Treated Chagasic Patients Revealed by Xenodiagnosis and Polymerase Chain Reaction

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Polymerase chain reaction (PCR) was compared with xenodiagnosis performed 20 years after trypanocidal chemotherapy to investigate parasite clearance. Eighty-five seropositive individuals for Chagas disease presenting a positive xenodiagnosis were treated with specific drugs; 37 in the acute phase and 48 in the chronic phase. Fifteen chronic asymptomatic patients received a placebo. Treatment in the acute phase led to PCR negative results in 73% of the cases, while xenodiagnosis was negative in 86%. In the chronic phase, PCR was negative in 65% of the patients and 83% led to xenodiagnosis negative results. Regarding the untreated group (placebo), 73% gave negative results by xenodiagnosis, of which 36% were positive by PCR. Individuals that were considered seronegative (n=10), presented unequivocally negative results in the PCR demonstrating the elimination of parasite DNA. Seventeen individuals had their antibodies titers decreased to such a level that the final results were considered as doubtful and 16 of them presented negative PCR. The molecular method represents a clear advantage over conventional techniques to demonstrate persistent infections in Chagas disease patients that underwent chemotherapy.

Key words: *Trypanosoma cruzi* - kinetoplast DNA - Chagas disease - PCR - treatment

The persistence of *Trypanosoma cruzi* in chronic lesions, revealed by molecular methods, supports the pathogenesis of Chagas disease mediated by presence of the parasite (Jones et al. 1993, Tarleton et al. 1997, Olivares-Villagómez et al. 1998, Añez et al. 1999). In fact, until recently, the current view was that auto-immune phenomenon was the major cause of clinical manifestations of the disease (Cunha-Neto et al. 1995, 1996). Probably the auto-immune component is a parasite-induced disorder (Tarleton & Zhang 1999). Therefore, etiologic treatment should focus on clearance of parasites. Most human infections with *T. cruzi* are only detected in the chronic phase for which

treatment is still controversial. Since conventional serological tests remain positive for years after successful therapy, a simple and safe procedure to assess cure is needed (Andrade et al. 1988, Gazzinelli et al. 1988). Negative xenodiagnosis and/or haemocultures have been used as evidence of effective treatment; however, these methods are time-consuming, laborious, uncomfortable for patients, and lack sensitivity (Brenner et al. 2000). A complement mediated lysis (CoML) test has been proposed to monitor treatment (Galvão et al. 1993), but the requirement for living trypomastigotes limits its use.

Preliminary results revealed the potential use of polymerase chain reaction (PCR) assay to monitor cure in patients undergoing specific treatment (Britto et al. 1995). Therefore, we investigated the use of PCR by comparing the results with xenodiagnosis, for evaluating cure in a group of 85 initially xenopositive patients from two Brazilian endemic areas who received trypanocidal treatment in an average of 20 years ago.

A randomized trial of trypanocidal drug versus placebo treatment in Chagas disease patients

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was carried out in two Brazilian endemic areas in the 70s, at the Department of Tropical Medicine, University of Brasília, Federal District of Brazil (Silveira 2000). In that study 236 patients were enrolled, 111 in the acute phase and 125 in the chronic phase, presenting at least two out of three positive serologic tests for *T. cruzi* infection and positive results by xenodiagnosis. Their clinical state was determined through physical exam, conventional electrocardiography and thoracic radiography. All the individuals examined were diagnosed as unquestionably chagasic. The treated chronic patients, both who received trypanocidal drug or placebo were asymptomatic. These same areas were revisited 20 years afterwards and 99 patients could not be recruited. Eighteen had died, 50 had moved from the districts and no information could be gathered from 31 individuals. From the 137 remaining patients (40 treated in the acute phase, 58 treated in the chronic phase and 39 that received placebo), a subset of 100 individuals (37 treated in the acute phase, 48 treated in the chronic phase and 15 that received placebo) was selected for the present study. They were all inhabitants of two distinct geographic areas: 49 from São Felipe (State of Bahia) and 51 from Mambai (State of Goiás), where vector transmission has been interrupted for more than 15 years. Patients were treated with nifurtimox (7-8 mg/kg/day, 60-90 days) or benznidazole (5-6 mg/kg/day, 30-60 days) (Silveira 2000).

The patients were followed-up by xenodiagnosis in an average of 20 years after treatment (range = 7-35 years), as previously described (Marsden et al. 1979). Serology was performed by indirect immunofluorescence (IIF), indirect hemagglutination (IHA) tests and enzyme-linked immunosorbent assay (ELISA). Patients were considered positive when their sera reacted in two of these tests at a dilution of at least 1:80, 1:32 and 1.2 times higher than the cut off, respectively. Doubtful results corresponded to titers of 1:20 to 1:40 in the IIF, 1:8 to 1:16 in the IHA and results of 0.9 to 1.1 times higher than the cut off in the ELISA. PCR amplification of the variable region of the minicircle molecule was used to detect the presence of parasite mitochondrial DNA (kinetoplast DNA) in whole blood, as described elsewhere (Britto et al. 1993, Wincker et al. 1994). All the reactions were performed in duplicates and samples considered to be negative in the PCR assay with *T. cruzi* specific primers, were further tested with human β -globin gene primers, to discard eventual inhibitors to the PCR test (Wincker et al. 1994). Furthermore, to enhance the sensitivity of detection of the amplified products, hybridization experiments were performed in all negative samples

and controls, using kinetoplast DNA from the Y strain MHOM/BR/00/Y (*T. cruzi*-II) as a radiolabeled molecular probe. No samples turned out to be positive after the hybridization step. Statistical analysis were performed with a statistical program of the EPI-INFO package version 6.0 using the Chi-square and Fisher's exact tests.

A total of 100 blood samples were analyzed: 85 from patients post-treatment for acute (n=37), or chronic indeterminate (n=48) Chagas disease, and 15 from the chronic chagasic asymptomatic patients that received placebo. Table shows the results obtained with xenodiagnosis and the PCR test performed simultaneously with both groups of treated patients and the placebo group. Treatment in the acute phase resulted in conversion of xenodiagnosis to negative in 32/37 (86%) cases. The PCR was positive in 5/32 xeno-negative individuals plus all of the xeno-positive patients. Of the treated patients in the indeterminate chronic phase, 40/48 (83%) had become xeno-negative, and PCR was positive in 9/40 of these and in all the xeno-positive patients. Overall, PCR was positive in 27% (10/37 in the acute phase) and 35% (17/48 in the chronic phase) of treated patients for acute or chronic phase, respectively.

In the untreated group (placebo), 11 out of 15 individuals were negative by the xenodiagnosis method (73%), of which 36% were positive by PCR assay. All 4 xeno-positive patients had positive PCR results. The low sensitivity of PCR (53%) and xenodiagnosis (27%) in this group suggests a scarce parasitaemia. These data reinforce the higher sensitivity of the PCR when compared to xenodiagnosis, and suggest its adoption as gold standard for parasitologic cure.

Of the 27 patients that were treated in the acute phase and presented negative results by PCR, 5 showed a doubtful serology 20 years afterwards and 7 had negative serological results. Considering the patients treated in the chronic phase, nega-

TABLE

Polymerase chain reaction (PCR) results in relation to xenodiagnosis in samples from treated chagasic patients and placebo group (untreated chronic asymptomatic controls). The column Xeno – and PCR + (bold) represent the advantage of the molecular technique over the traditional parasitological diagnosis

	(N)	Xeno +		Xeno –	
		PCR –	PCR+	PCR –	PCR+
Acute phase	37	0	5	27	5
Chronic phase	48	0	8	31	9
Placebo group	15	0	4	7	4

tive serological results were evidenced in 3 and 12 showed doubtful results. No patients with an actual negative serology presented a PCR positive result (n=10). However, one patient that was treated in the chronic phase presenting a doubtful serological result showed *T. cruzi* DNA amplification. Indeed, the negative serology was a good marker for cure in this study since all of them were PCR negative.

At present, nifurtimox or benznidazole are indicated for the acute *T. cruzi* infections, for infected children and also for chronic chagasic patients that are either in the indeterminate form or in the early symptomatic stage (Ministério da Saúde 1997). However, there are many difficulties involved in the evaluation of the efficacy of parasitocidal treatment mainly during the chronic phase of Chagas disease (Brener 1984).

PCR presented a higher positivity than the xenodiagnosis method for detecting parasites in all studied groups, although it was statistically significant only in the group comprised of chronic patients that received trypanocidal drug (17 PCR positive results versus 8 xenopositive individuals in 48 patients; $\chi^2=4.34$, $p=0.03$). For instance, 13% of treated patients in the acute Chagas disease gave a positive PCR result while the xenodiagnosis had become negative. In the chronic phase, 18% of the cases were under-diagnosed by the xenodiagnosis procedure. These patients that are xeno-negative and PCR-positive should be followed-up, in order to demonstrate the role of the presence of circulating DNA, shown by PCR, in the evolution of symptoms. Regarding the untreated group, PCR presented a negative result in 47% of the cases. This rate may reflect the intermittent parasitaemia that is usually found in chronic chagasic patients, even in those who were treated and did not present parasitological cure.

The interpretation of PCR may be raise controversial. A positive result may reflect the detection of intact parasites or circulating DNA. However, Tarleton and Zhang (1999) described that after the intra-muscular injection of large quantities of kDNA, PCR was positive only for two days after the injection and not thereafter. The authors discuss that this study strongly supports the hypothesis that parasite DNA detected by PCR is derived from intact, extracellular or recently lysed parasites.

A negative post-treatment PCR result may be indicative of the absence of parasite DNA at that moment. In our scenario, as only one point in time was analyzed, a negative result could not be assumed as definitive cure. In the present study, all the individuals that became negative by serological tests performed 20 years after chemotherapy,

also demonstrated the elimination of circulating DNA by the PCR method (data not shown). The agreement of negative results between serology and PCR, is probably indicative of cure. Although these data indicate that PCR is more reliable and sensitive than xenodiagnosis to assess cure after specific treatment, the use of a single assay, as performed in this setting, is not sufficient to reach a final conclusion on parasite DNA clearance in post-treatment patients. Follow-up studies with a continuous screening of individuals, using the molecular technique plus serology, should be carried out.

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