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POLYMERASE CHAIN REACTION (PCR) AS A LABORATORY TOOL FOR THE EVALUATION OF THE PARASITOLOGICAL CURE IN CHAGAS DISEASE AFTER SPECIFIC TREATMENT

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Abstract The evaluation of the treatment for chronic Chagas disease faces the absence of any clear-cut criterion of cure. The low degree of parasitemia and the persistence of positive immunologic reactions represent some of the difficulties involved in addressing therapeutic efficacy. Our aim was to define whether PCR could be used as a laboratory method for evaluating cure in Chagas disease after specific treatment. We tested the utility of PCR amplification of the variable regions of minicircles from *Trypanosoma cruzi* kinetoplast DNA, in 76 xenopositive chronic Brazilian patients who have been treated with benznidazole in Mambai (Goias State) and São Felipe (Bahia State). We observed a positive amplification result in only 25 out of 76 treated patients (33%). Therefore, the performance of one single PCR after therapy revealed parasite clearance in 67% of the treated individuals, while xenodiagnosis was negative in 84%. These observations suggest that PCR is the most sensitive technique available for direct detection of *T. cruzi* in chagasic patients and that it can be a very useful instrument for the follow-up of patients after specific chemotherapy. In this sense, we are now developing a quantitative approach based on the use of fluorogenic probes and real-time measurement of the amplification reaction (*TaqMan* technology) in order to precisely estimate the parasite load in chronic chagasic patients before and after treatment. This may be the basis for the future establishment of reliable criteria of cure for patients undergoing therapy.

Resumen ***Reacción en cadena de la polimerasa (PCR) como herramienta de laboratorio para la evaluación del tratamiento de la enfermedad de Chagas.*** La evaluación del tratamiento de la enfermedad de Chagas crónica tropieza con la falta de criterios seguros para decidir la curación/no curación de la enfermedad. Una parasitemia de bajo nivel y la persistencia de reacciones inmunológicas positivas representan una de las dificultades halladas para juzgar la eficacia terapéutica de los medicamentos ensayados. El objetivo de nuestra investigación fue definir si la PCR (*Polymerase Chain Reaction*) podría ser utilizada como método de laboratorio para confirmar la curación, después de tratamientos específicos. Ensayamos la utilidad de la amplificación por PCR de regiones variables de los minicírculos del DNA cinetoplástico del *Trypanosoma cruzi*, proveniente de enfermos chagásicos brasileños que habían sido tratados con Benznidazole en las poblaciones de Mambas (Goias) y San Felipe (Bahía). Observamos amplificación positiva en 25/76 pacientes (33%). Por lo tanto la realización de una sola PCR después del tratamiento reveló la erradicación del parásito en 67% de los individuos tratados, mientras que el xenodiagnóstico fue negativo en 84%. Estas observaciones sugieren que la PCR es el método más sensible para la detección directa del *T. cruzi* en pacientes chagásicos y que puede constituir un instrumento muy útil para el seguimiento de los chagásicos después de la quimioterapia específica. En este contexto estamos desarrollando un examen cuantitativo basado en el uso de sondas fluorogénicas y medidas a tiempo fijo de la velocidad de la reacción de amplificación (tecnología *TaqMan*) a fin de estimar con precisión la carga parasitológica en chagásicos crónicos antes y después del tratamiento. Ello puede constituir la base para establecer en el futuro criterios confiables para la curación de los pacientes sometidos a la quimioterapia específica.

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

In humans, Chagas disease caused by infection with the protozoan parasite *Trypanosoma cruzi* induces an acute phase with patent parasitemia followed by a life-long chronic phase characterized by low levels of circulating parasites and scarce tissue parasitism[1]. Disease pathogenesis involves immune mechanisms that control the exponential growth of parasites in the early phase of infection, followed by a sustained immune response that keeps a subpatent parasitemia in the chronic phase[2]. This host immunological response prevents re-infection upon challenge but cannot eradicate ongoing infection.

A wide spectrum of clinical manifestations develops within 10 to 20 years after the acute phase of the disease[1]. Surprisingly in the chronic phase, blood and tissue parasites are extremely scarce. The role of the presence of *Trypanosoma cruzi* in the pathogenesis of chronic Chagas disease was, and still is, a subject of dispute

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among researchers and clinicians. During many years the traditional detection techniques could not demonstrate the presence of parasites in damaged tissues. In fact, until some years ago the current view was that autoimmune phenomena were the major cause of clinical manifestations and could possibly be sustained without the need of a constant presence of the parasite[3, 4]. Although the role of the autoimmune response has been recently confirmed[5, 6], the crucial role played by the parasite has also been evidenced[7-10]. With the development of molecular techniques such as the polymerase chain reaction (PCR), it became possible to show that parasites do play an active role in the disease pathogenesis. These data demonstrate the importance that should be given to the specific etiologic treatment of chronic infections, particularly at early stages, in order to prevent the progression of the disease.

The Brazilian Ministry of Health has determined that it is appropriate to treat cases of Chagas disease, provided patients namely in either the acute, indeter-minate or chronic phases, are not presenting extremely severe symptoms of cardiopathy or digestive tract involvement. Many drugs have been tested for efficacy against Chagas disease, in experimental animals and in humans. However, although the first trials of therapy of Chagas disease date back to the 30's, there are still no ideal drugs and those in use such as nifurtimox and benznidazol, are well known for their undesirable side effects. This has limited their use to specific situations, such as: acute cases, accidental and congenital infections, disease reactivation in immunosuppressed patients and in clinical investigations with selected patients still in early stages of the disease. On the other hand, recent results have suggested the convenience of benznidazol treatment of seropositive children as part of public health programs[11, 12]. Nowadays benznidazol is commonly used and different criteria for doses are regulated by the Brazilian Ministry of Health.

It is therefore fundamental to be able to monitor the efficacy of the several therapeutic alternatives and to establish reliable criteria to control the cure of patients. The criteria typically followed are: clinical, parasitological and serological parameters. Traditional parasitological approaches, such as hemoculture and xenodiagnosis are labor-intensive and of low sensitivity[13, 14]. Immunological methods, such as lytic antibodies[15, 16] and "antigen-trypomastigote" ELISA[11], although promising, has not yet been incorporated in routine assays. The criteria for therapeutic monitoring are difficult to evaluate in all cases due to particularities in the natural history of the infection. Also, the utility of the criteria depends on the stage of the disease. The evaluation of cure after treatment of acute phase infections is not difficult since parasitemia is high and subsides within one month of drug therapy.

The major problem of treatment evaluation occurs during the chronic phase when parasitemia is extremely low and it is difficult to detect *Trypanosoma cruzi* even before the treatment. Our approach to this problem has been the direct parasitological detection of *Trypanosoma cruzi* based on the amplification of specific parasite nucleotide sequences by the polymerase chain reaction (PCR), a technology developed in our Institute[17-22] and which we have already demonstrated to be useful in the evaluation and follow-up of therapy[23]. Parallel, immunological tests should be conducted since it is expected that there will be a decrease in the titers of anti-*T. cruzi* antibodies, if treatment is effective. Up to date, no trial research has been conducted using molecular techniques to investigate parasitological cure after treatment in Chagas disease concomitantly with traditional immunologic methods.

Our Institute has, in conjunction with the Tropical Medicine Department of the University of Brasilia (Brazil), evaluated 76 chronic patients that were been treated 20 years ago in Mambai (Goias State) and São Felipe (Bahia State), all of them being xenopositive before the treatment. The aim was to define whether PCR could be used as a laboratory method for evaluating cure in Chagas disease after specific treatment. In this sense, we collected 10 ml of blood from each patient mixing it with the same volume of Guanidine-HCl 6M/EDTA 0.2M (GEB lysate buffer)[24]. The mixture was boiled for 15 minutes, in order to decatenate the mitochondrial DNA kinetoplast (kDNA) network of the parasite[17], allowing a homogeneous distribution of minicircle molecules throughout the blood lysate. One hundred microliters of the GEB lysate were submitted to phenol: chloroform extraction and sodium acetate/ethanol precipitation of the DNA. The final pellet was resuspended in 50 microliters of double distilled water and 7.5 microliters were used in the PCR reaction.

The PCR was mediated through the use of oligonucleotides that anneal to the conserved region of the minicircle molecule, resulting in an amplification of the variable region, giving rise to a fragment of 330 base pairs. A hot-start technique was used in order to enhance the specificity of the assay. The PCR products were submitted to agarose gel electrophoresis, ethidium bromide staining and visualization under UV light.

Of the 76 treated patients, PCR was positive in 25, which represents almost 33%. Therefore, the performance of one single PCR after treatment revealed clearance of *T. cruzi* in 67% of the treated patients. A more careful evaluation should be carried out by analysing these patients in a follow-up survey of some years, to show that the treatment was really effective.

The methodology of PCR detection of specific sequences has recently been dramatically improved with the development of the so-called *TaqMan* technology, an automated, quantitative approach based on the use of

fluorogenic probes and real-time measurement of the amplification reaction[25-27].

Our Institute has recently acquired an ABI Prism 7700 Gene Detection System which allows the use of this quantitative technology for the automated analysis of 96 samples in real time. We developed *T. cruzi*-specific *TaqMan* fluorogenic probes to be used in the detection of both the constant and variable region sequences of kinetoplast DNA minicircles[21], in order to precisely measure the parasite load in chronic chagasic patients before and after treatment, a necessary basis for the future establishment of reliable criteria of cure for patients undergoing therapy.

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