

RESEARCH NOTE

Polymerase Chain Reaction Detection: New Insights into the Diagnosis of Chronic Chagas Disease

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The chronic phase of Chagas disease is characterized by very low levels of parasitemia and high titers of antibodies directed against *Trypanosoma cruzi* antigens. The laboratorial diagnosis in this phase is performed mainly by serological assays or indirect parasitological methods where the parasite population can be amplified through xenodiagnosis and blood culture. Although highly specific, xenodiagnosis has limited sensitivity, the parasites are detected in only 20-50% of individuals known to be infected (WHO 1991 *Control of Chagas Disease*, Report of a WHO Expert Committee, p. 97), resulting in many false negative results. Hemoculture has not been used frequently also due to its low positivity. Regarding serological techniques, the specificity of these assays has been questioned, due to the frequency of infections with other trypanosomatids that circulate in the same geographic area of *T. cruzi* and are responsible for cross-antigenicity and false positive results (AW Ferreira 1992 Tests for Chagas disease

serodiagnosis: a review, p. 179-193. In S Wendel, Z Brenner, ME Camargo & A Rassi (eds) *Chagas Disease (American Trypanosomiasis): its Impact on Transfusion and Clinical Medicine*, ISBT, São Paulo). These limitations explain the interest in a direct and sensitive method for the diagnosis of Chagas disease, such as the polymerase chain reaction (PCR).

Studies have been carried out using *T. cruzi*-specific PCR assays for the detection of parasite DNA in blood samples. Either kinetoplast DNA (kDNA) or nuclear satellite DNA has been used as target sequences envisioning the potential usefulness of the PCR technology for the diagnosis of chronic Chagas disease (N Sturm et al. 1989 *Mol Biochem Parasitol* 33: 205-214, DR Moser et al. 1989 *J Clin Microbiol* 27: 1477-1482, H Avila et al. 1991 *Mol Biochem Parasitol* 48: 211-222, C Diaz et al. 1992 *Am J Trop Med Hyg* 46: 616-623, JM Requena et al. 1992 *Mol Biochem Parasitol* 51: 271-280, G Russomando et al. 1992 *J Clin Microbiol* 30: 2864-2868). However, since the concentration of trypanosomes in the blood of chagasic individuals varies enormously on different regions, the efficiency of such tests needs to be further investigated using blood samples from diverse endemic areas with different epidemiological profiles, before any definitive conclusions about the sensitivity of the PCR technique can be reached.

When kDNA is used as the target for PCR amplification, the kinetoplast network must be cleaved in order to enable the homogeneous distribution of minicircle molecules in blood samples. The use of the chemical nuclease copper/phenanthroline and radioactive detection of the amplified products, dramatically improved the sensitivity of the method, detecting as few as one parasite in 20 ml of blood (see Avila et al. 1991 *loc. cit.*), allowing the efficient diagnosis of chronic Chagas disease in human patients (H Avila et al. 1993 *J Clin Microbiol* 31: 2421-2426). The chemical cleavage protocol, however, introduces the need for extra manipulation of the samples and therefore increases the possibility of cross contamination and false positive in PCR-based diagnostic protocols.

We have found that physical cleavage of kDNA network can be efficiently accomplished by simply boiling the guanidine-EDTA-blood lysates (GEB lysates, see Avila et al. 1991, *loc. cit.*). This finding allowed us to develop a simple, rapid, reliable and less expensive protocol for the PCR-based detection of *T. cruzi* useful in diagnostic, clinical and epidemiological studies. Using the hot-start PCR technique with oligonucleotides complementary to the *T. cruzi* highly conserved blocks in the kDNA minicircle sequence, a 330 bp fragment

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containing the variable regions of the molecule can be amplified. Using this approach we reached the same sensitivity previously described by Avila et al. (1991, *loc. cit.*), detecting one parasite in at least 20 ml of GEB lysate (C Britto et al. 1993 *Mem Inst Oswaldo Cruz* 88: 171-172).

In order to evaluate the performance of the hot-start PCR protocol for Chagas disease diagnosis, our group has undertaken a series of studies in different endemic regions with particular epidemiological characteristics. These assays were conducted in the states of Minas Gerais (Virgem da Lapa), Paraíba and Piauí (P Wincker et al. 1994 *Am J Trop Med Hyg* 51: 771-777, C Britto et al. 1995 *Exp Parasitol* 81: 462-471, ACV Junqueira et al. 1996 *Trans R Soc Trop Med Hyg* 90: 129-132), traditional endemic areas of Chagas disease, and in the Amazonas Basin (JR Coura et al. 1995 *Rev Inst Med Trop São Paulo* 37: 415-420) where *T. cruzi* is enzootic. In the four states, seroprevalence is in the range of 6-13.3%. Detection of circulating parasites in some seropositive individuals was performed by xenodiagnosis and PCR amplification of minicircle kinetoplast DNA. Both assays showed higher sensitivity in chagasic patients from endemic regions. In fact, in Minas Gerais, Paraíba and Piauí, xenodiagnosis showed, respectively, 24.5%, 13% and 34.2% positivity, and PCR amplification detected parasite DNA in 96.5% (Minas Gerais), 44.7% (Paraíba) and 59.4% (Piauí) of the seropositive individuals. In Amazonas both xenodiagnosis and PCR revealed a positivity of 2.4% and 10%, respectively, suggesting a low level of parasitaemia in that region.

Patients receiving specific treatment represent a second problem for which new detection techniques are expected to supply important solutions. Few drugs against *T. cruzi* are currently available, and their efficiency is questionable owing to the lack of a reliable system for assessing cure (SL Castro 1993 *Acta Trop* 53: 83-98). The conventional serology is particularly ineffective in this situation, since the fading of the humoral response occurs long after the elimination of the parasite (Z Brener & AU Krettl 1990 Immunology of Chagas disease, p. 247-261. In DJ Wyler, *Modern Parasite Biology: Cellular, Immunologic and Molecular Aspects*, WH Freeman and Co., New York). Immunological methods, such as lytic antibodies (LMC Galvão et al. 1993 *Trans R Soc Trop Med*

87: 220-223) and ELISA using trypomastigote antigens (ALSS De Andrade et al. 1996 *The Lancet* 348: 1407-1413), although looking promising, have still not been incorporated in routine assays.

The performance of the PCR detection method was tested on treated individuals attending the Evandro Chagas Hospital, Fiocruz, Rio de Janeiro. The results demonstrate a positive amplification signal detection in only 9 out of 32 treated patients who remained reactive when analyzed using classical serology (C Britto et al. 1995 *Parasitology* 110: 241-247). These preliminary assay suggest that PCR can be a very useful tool in the evaluation and follow-up of therapy. It is therefore fundamental to be able to monitor the efficacy of the different therapeutic alternatives to Chagas disease and to establish reliable criteria for the control of cure.

The methodology of PCR detection of specific genes and sequences has recently been dramatically improved with the development of the *TaqMan* technology an automated, quantitative approach based on the use of fluorogenic probes and real-time measurement of the amplification reaction (KJ Livak et al. 1995 *PCR Meth Appl* 4: 357-362, CA Heid et al. 1996 *Genome Research* 6: 986-994). In this sense, our prospects are to develop *T. cruzi*-specific *TaqMan* fluorogenic probes to be used in the detection of both the constant and variable region sequences of kinetoplast DNA minicircles in order to measure the parasite load in chronic chagasic patients before and after treatment, a necessary basis for the future establishment of reliable criteria of cure of patients undergoing therapy.

Another contribution of PCR technology is related with paleoparasitology studies allowing the identification of ancient DNA from archaeological material. Recently, we were able to recover *T. cruzi* DNA in tissue from Chilean mummies dated from 2,000 years BP to 1,400 AD, from the Atacama desert. Nowadays, the city of San Pedro de Atacama is considered an endemic area for Chagas disease. Six mummified tissues were tested and amplification of the conserved region of the minicircle molecule, generating a fragment of 120 bp, was achieved in four mummified bodies. Further hybridization experiment with *T. cruzi* specific molecular probe, proved that the amplified products correspond to genetic fragments of the parasite.