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PCR and Sero-Diagnosis of Chronic Chagas' Disease

Biotechnological Advances

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ABSTRACT

In the acute phase of Chagas' disease, when the parasitemia is high, diagnosis can be easily made using conventional parasitological methods. During the chronic phase, due to the low parasitemia, diagnosis is performed mainly by immunological methods. Conventional serological techniques are limited by cross-reactivity with other parasitic diseases, non-standardization of reagents, and the diversity of technical procedures. Methods are being developed to improve the sensitivity and specificity of diagnosis using molecular approaches. PCR-based detection systems and the use of recombinant antigens in ELISA are the most promising.

Index Entries: *Trypanosoma cruzi*; Chagas' disease; diagnosis.

INTRODUCTION

Chagas' disease or American trypanosomiasis, is a major health problem in Latin America where some 16–18 million people are infected with the causative agent, *Trypanosoma cruzi* (*T. cruzi*) (*1*). There is a high morbidity among these infected individuals, because there is no vaccine or safe chemotherapy currently available for prevention or treatment of this disease.

T. cruzi is a protozoan parasite that infects various mammals, including humans, and is transmitted by an insect vector, the reduviid bug. Under natural conditions, a hematophagous reduviid bug will ingest blood containing parasites from an infected individual. The *T. cruzi* then differentiate and multiply within the digestive tract of the vector. The infective metacyclic trypomastigotes are eliminated in the feces and can

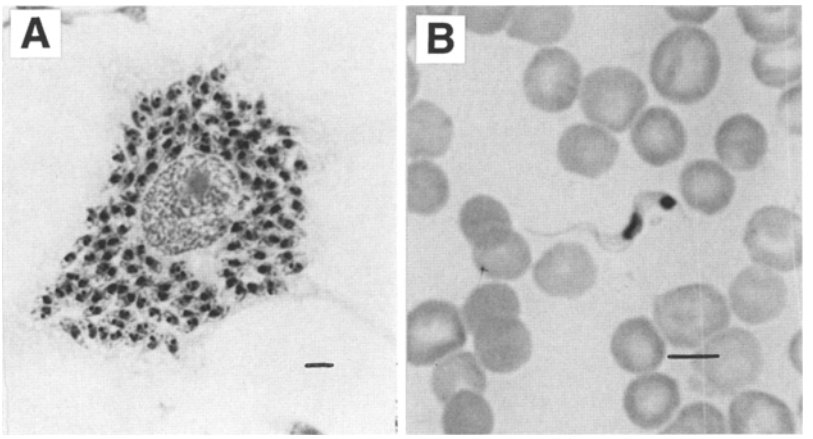

Fig. 1. **(A)** In vitro biological cycle of *Trypanosoma cruzi* Y strain: amastigote in Vero cells; 72 h of culture; **(B)** trypomastigote in the peripheral blood (Giemsa stain). Bars = 6 μm.

infect the human host by contamination of skin lesions or intact mucous membranes. However, *T. cruzi* may bypass the vectors and be transmitted to man by a number of alternative mechanisms:

1. blood transfusion;
2. congenital transmission;
3. accidental laboratory infection;
4. organ transplantation; and
5. oral.

Whatever the route of infection, the trypomastigotes can enter a variety of cell types, primarily muscle cells and macrophages. Upon entry, they differentiate into amastigotes which undergo multiplication before changing back into trypomastigotes (Fig. 1). The trypomastigotes disrupt the host cells, are liberated, and can circulate in the blood for a variable period of time before infecting other cells and repeating the process. At some stage, the life cycle is continued by another reduviid bug ingesting blood from an infected individual *(2)*.

In the acute phase of Chagas' disease, a large number of parasites circulate in the bloodstream. During this period, diagnosis is based upon detection of these organisms by direct parasitological methods (Table 1). In contrast, chronic Chagas' disease, which follows the resolution of the acute infection, is characterized by levels of circulating parasites far below the threshold for microscopic detection and by high titers of antibodies directed against *T. cruzi* antigens. Diagnosis in this phase is based primarily on serological methods or indirect parasitological methods: xenodiagnosis and blood culture. Although highly specific, xenodiagno-

Table 1
Parasitological Methods for Diagnosis of Chagas' Disease According to WHO *(1)*

Methods	Type of laboratory[a]	Percentage sensitivity[b]	
		Acute stage	Chronic stage
Direct			
Thin blood smear	A/B	<60	<10
Thick blood smear	A/B	<70	<10
Fresh blood examination	A/B	80 - 90	<10
Strout	A/B	90 - 100	<10
Buffy coat on slide	A/B	90 - 100	<10
Indirect			
Xenodiagnosis	B	100	20 - 50
Blood Culture	B	100	20 - 50

[a]A, Health center laboratories located in areas at risk of vectorial and nonvectorial transmission (the infrastructure is that from the first level of medical care upwards). B, Specialized laboratories for parasitological diagnosis *(1)*.

[b]As compared to xenodiagnosis for the acute stage of the infection and to serological diagnosis for the chronic stage.

sis has limited sensitivity, because parasites are detected in only 20–50% of individuals known to be infected (Table 1), resulting in many false negative results. Blood cultures, together with serological methods, are used to assess cure after treatment, but the sensitivity is again about 20–50% (Table 1). Accurate diagnosis at this stage is important for the individual and also for epidemiological reasons, because transmission by transfusion of blood donated by infected persons is common in many endemic areas *(3,4)*. However, accurate diagnosis is not easy, particularly in the chronic asymptomatic form of the infection, during which contradictory results have been described. The reason for variation results between different serologic assays is primarily a question of antigen quality (crude or partially purified parasite extracts). On the other hand, the anti-

gen complexity of *T. cruzi (5)* as well as the heterogeneity of parasite populations *(6,7)* must also be taken into consideration, since antigens "particular" to different strains of *T. cruzi (8)* have been detected. A brief evaluation of immunological methods used to detect antibodies against *T. cruzi* in the chronic phase and the prospects for more specific diagnosis are discussed below.

IMMUNOLOGICAL METHODS

Chronic Chagas' disease is usually diagnosed by detecting IgG that binds specifically to *T. cruzi.* Several serological tests are widely used in Latin America, such as complement fixation (CF), indirect hemaglutination (IHA), indirect immunofluorescence (IIF), and ELISA. These conventional serological assays are used for individual diagnosis and for screening donated blood, as well as in epidemiological studies. However, a persistent problem with these conventional serologic assays has been the occurence of false-positive results. These reactions typically occur in patients with other parasitic diseases, such as *Trypanosoma rangeli* infection, visceral and cutaneous leishmaniasis, syphilis, toxoplasmosis, hepatitis, systemic lupus erythomatous, hanseniasis, schistosomiasis, rheumatoid arthritis, paracoccidioidomycosis, and mononucleosis *(9–12).* Because of this problem, the World Health Organization (WHO) recommends that serum specimens must be tested in three conventional assays before being accepted as positive. This latter approach carries with it an enormous logistical and economic burden, especially for a blood bank. Even when the blood banks use three different serological tests to reach a diagnosis, the amount of blood discarded that could be transfused, if there was a safe test, is significant. As an example, in the largest blood bank in São Paulo, Brazil, where three serological tests for antibodies to *T. cruzi* are used (IIF, IHA, and CF) 3.4% of donated units are discarded due to reactivity in these assays. However, as many as two thirds of these may be false-positives but must be discarded because of inconsistent test results *(13).*

The use of crude or partially purified extract of parasite, as well as the different procedures in different laboratories, leads to inconsistent results. In addition, autoantibodies can give false positive reactions. Recently, Velazques et al. *(14)* analyzed 201 sera with autoantibodies from Finnish and Swedish individuals with no evidence of *T. cruzi* infection. Sera were grouped according to types of autoantibodies present: anti-mitochondrial antibodies; anti-reticulin; anti-nuclear; anti-intermediate filaments; anti-glomerular basement membrane (from patients with Goodpasture's syndrome); anti-smooth muscle, and anti-tubulin. By the IIF test, using epimastigotes as antigen, these researchers verified that 15% of these sera were positive at a titer ≥ 1:250. Usually a single serum exhibited a mixed

staining pattern (flagellar, nuclear, coarse or fine granular cytoplasmic, surface membrane, kinetoplast), indicating the presence of antibodies of different specificity. Similar results were obtained with sera of patients with Chagas' disease from Costa Rica and Guatemala. These data are consistent with previous observations of Minoprio et al. *(15)*, and suggest that serum autoantibodies directed against shared antigens may arise during various chronic-inflammatory conditions. The results show that autoantibodies are a potential source of false-positive reactions in tests, using epimastigotes as antigen. This must be taken into account, e.g., in the differential diagnosis of chronic myocarditis and in screening for *T. cruzi* in blood-banking.

These facts show a need for standardization of the procedures and for better-defined molecular reagents. In addition, exact levels of sensitivity and specificity are particularly necessary in blood-bank control, where false-negatives will lead to disease transmission, and false positives will lead to excessive discarding of useful blood. Because procedures for making a specific parasitological diagnosis (xenodiagnosis or blood culture) are insensitive, laborious, and take several weeks to complete, a number of research laboratories have tried to develop tests with the sensitivity of the conventional assays, but which are also specific and simple to perform.

With this aim, several research groups have examined the possibility of using monoclonal antibodies (MAbs) in differential diagnosis of *T. cruzi* from other parasites. The MAb anti-GP-72, which is species-specific to *T. cruzi (16)*, was used in a competitive immunoenzymatic assay using as antigen the component 5 present in the membrane of epimastigote and bloodstream trypomastigote forms *(17)*. This assay showed an elevated sensitivity of about 96.6% in the chronic phase of infection and no false-positive reaction was observed in sera of normal individuals or those bearing other parasitic diseases. Because of its simplicity, specificity and sensitivity, this test was recommended for diagnosis and screening of chronic Chagas disease, particularly in areas where concomitant infections are unknown or suspected *(18)*. A competitive ELISA assay using a 25-kDa polypeptide and a MAb that recognizes it has also been proposed *(19)*. This polypeptide is present in all developmental stages of *T. cruzi* and was recognized by sera of symptomatic and asymptomatic patients, independently of their geographic area *(20)*. MAbs have also been used to detect circulating antigens during the chronic phase. The characterization of an 85-kDa secretory antigen of *T. cruzi* found on the surface of mammalian stages of the parasite was recently described by Ouaissi et al. *(21)*. Using a MAb that binds to this antigen, they developed an ELISA to detect the antigen in serum samples from 80 chronic-phase patients (58 Bolivian and 22 Argentinian). The 85-kDa antigen was detected in 98% of Bolivian sera and in 81% of sera from Argentina.

According to Bergquist *(22)*, although MAbs are exclusively directed against one epitope only, cross-reactions may occur. This is because epi-

topes are not unique, but can be shared between different microorganism. In addition, the production of MAbs exclusively reacting with one antigen, is presently more difficult than generating specific antigens by recombinant DNA techniques. This is because the selected epitope is exactly specified by its DNA sequence, whereas antibody reagents have to be chosen from an array of MAbs with different and, in principle, unknown specificities. Although the increasing number of MAbs available against several parasites, including *T. cruzi*, is overcoming this hurdle, the lack of field-applicable techniques continues to limit the routine use of serology in national control programs.

Attempts have been made to substitute conventional crude antigens by purified parasite proteins *(23,24)*. However, the problem in the case of *T. cruzi* is that such proteins are present in very limited quantities, and their purification by biochemical techniques is highly laborious. One way of resolving this problem is the use of genetic engineering, which permits greater quantities of defined antigens to be obtained.

PROSPECTS FOR SPECIFIC DIAGNOSIS

The problems with conventional assays (IHA, IIF, and ELISA), which were discussed in the previous section, may be overcome by using recombinant polypeptides containing specific *T. cruzi* epitope that elicit an immune response in the majority of chagasic patients. These antigens should be common to all known *T. cruzi* strains and should not cross react with sera from patients carrying other diseases. A good recombinant antigen should be easy to obtain through reproducible methodology and be used, preferentially, in conjunction with commonly employed laboratory techniques *(25)*.

Recombinant DNA technology has led to the molecular cloning of several genes encoding antigenic *T. cruzi* proteins. Cloned segments of *T. cruzi* genes have been used to produce portions of antigenic proteins in bacteria, and several of these, singly and in combination, have been used as target antigens in serodiagnostic assays. Most of the work has been directed toward development of assays for chronic Chagas' disease *(25,26–31)*.

In order to assess the sensitivity, specificity, and predictive values of some defined antigens—and their potential for diagnosis in blood banks—the TDR Joint Steering Committee on Chagas' disease recommended in June, 1989, the implementation of a double-blind multicenter study on a limited number of these reagents *(32)*. The laboratory of Chagas' disease of the Federal University of Goiás, Brazil, was selected as one of the reference laboratories. The serum samples were distributed to the participating laboratories to be processed by the routine technique of each laboratory, using as antigens the recombinant/synthetic proteins and the biochemically purified proteins (Table 2). The best 11 antigens (CRA, B13, H49, JL7, A13,

Table 2
Antigens Used in the Multicenter Double-Blind Study According to Moncayo & Luquetti *(32)*

Laboratory	Antigens	Technique
1	CRA	ELISA
2	GP-57/51	ELISA
3	NEUROAMINIDASE	IMMUNOBLOT
4	JL5/JL7/JL8/JL9	DAI*
5	GP-90	ELISA
6	B-12/B-13	RIA
7	Ag 1, 2, 7, 13, 26, 30, 36, 54	DBIA**
8	H-49, A-13	DBIA
9	A-1/A-4	ELISA

*Dot Array Immunoassay
**Dot Blot Immunoassay

JL5, Ag1, Ag2, Ag30, JL8, A4) had Kappa indexes equal or greater than 0.80, i.e., their serological reactivity showed an excellent agreement with the results of the reference laboratory in Goiás. They also showed high sensitivity and specificity *(32)*. Studies are being undertaken to develop some of the aforementioned antigens—or combinations of them—to produce better kits for screening in blood banks. Two recombinant antigens expressed in *Escherichia coli*, which display a repetitive-epitope structure, have been investigated *(29,33)*. One of the antigens, which has a 68-amino acid basic repeat, is located in the flagellum of the parasite (flagellar repetitive antigen [FRA]), whereas the other is distributed throughout the cytoplasm (cytoplasmic repeat antigen [CRA]) and has a 14-amino acid repeat *(33)*. These antigens give better results when used in combination rather than separately. A direct ELISA assay, which involves the use of peroxidase-labeled antigens to detect immune complexes, was developed by Krieger et al. *(34)* and compared with a conventional ELISA. The recombinant (CRA + FRA) ELISA was better than the conventional ELISA in the diagnosis of Chagas' disease, providing 100% specificity and sensitivity with all sera tested. When compared with conventional serologic

tests (IHA and IIF) the recombinant ELISA did not give rise to the false-positive results which were observed with the other two tests. Given these observations, and the fact that *T. cruzi* recombinant antigens are cheaper and safer to produce than live-parasite extracts, this system developed by Krieger et al. *(34)* should be of great value in improving the current diagnosis of Chagas' disease.

With regard to direct detection of the parasites in peripheral blood, the prospects are also promising. The new polymerase chain reaction (PCR) technology is being used for the diagnosis of several diseases, including chronic Chagas' disease. Diagnosis by PCR is based on the in vitro amplification of specific-nucleotide sequences present in the organism. Although the number of circulating parasites in the blood of persons with chronic Chagas' disease is extremely low, they can be detected by PCR-based assays, because their highly repetitive nuclear and kinetoplast DNA (kDNA) sequences can be used as targets for amplification. In order to understand the use of PCR with *T. cruzi*, the genomic complexity of this parasite will be briefly discussed.

Trypanosoma cruzi cells, like other kinetoplastids, contain a complex network of concatenated circular DNA molecules (termed kinetoplast DNA or kDNA) within their single mitochondrion. The mitochondrial DNA of this parasite represents about 20% of the total cellular DNA and is organized in a disk-shaped network where thousands of small interlocked minicircles make up 95% of this structure. Each minicircle is organized into four 120 base-pairs (bp) minirepeat conserved regions that are present in every molecule, and a variable region that can provide a source of population-and subpopulation-specific probes *(35–37)*.

Oligonucleotides derived from the conserved region of the *T. cruzi* DNA sequence have been used in PCR-based assays, in order to detect this parasite in human blood samples. Sturn et al. *(38)* have amplified two overlapping fragments from the minicircle DNA (amplification product = 83 and 122 bp) and one fragment covering the adjacent-variable regions (amplification product = 330 bp) (Fig. 2). The minimal amount of minicircle DNA required for detection by an oligonucleotide probe was 0.015 fg, which represents approx 0.1% of the minicircle DNA component of a single parasite. The amplification of these fragments worked equally well with kDNA from several strains of *T. cruzi* (CL, Peru, and Y). However, DNA from the closely related species, *T. rangeli*, also shows amplification of the conserved region fragments. No product was produced when *T. rangeli* kDNA was amplified with the variable region primer set. kDNA networks from several other kinetoplastid genera (*Crithidia lucilliae, Leptomonas collosoma, Herpetomonas miriadeanei, Endotrypanum* sp, *Blastocrithidia culicis*, and *Leishmania tarantolae*) showed no amplification product. These authors also showed that kDNA recovered from less than 10 trypanosomes in 100 µL of whole blood from an infected mouse could

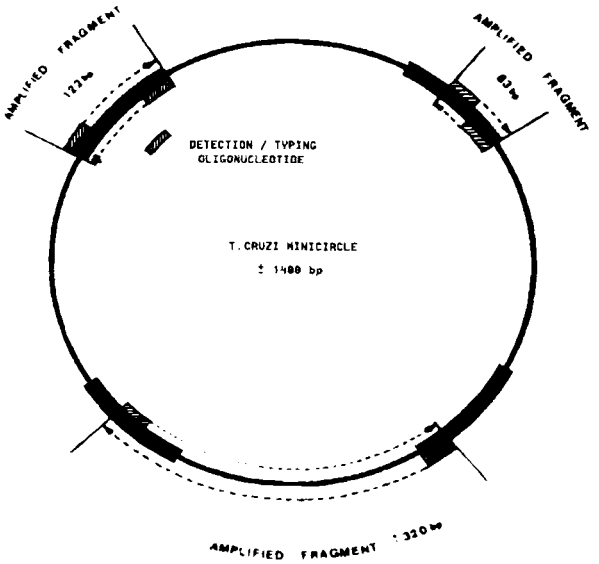

Fig. 2. *Trypanosoma cruzi* minicircle molecule showing the four conserved regions and the different PCR strategies for amplification of conserved and variable regions (photo courtesy of W. Degrave, reproduced by permission of Memórias do Instituto Oswaldo Cruz).

be used as a template for amplification. The results obtained by Sturn et al. *(38)* show that the absence of strain-specificity in the amplification of minicircle fragments implies that these products can be used as diagnostic markers for *T. cruzi*. Two refinements of the procedure were described by Avila et al *(39)* in a subsequent report. The first was preservation of human blood specimens at room temperature by mixing with a guanidine-EDTA buffer (GEB) and the second was the use of a chemical nuclease, 1,10-phenanthroline-copper ion (OP-Cu^{2+}), to liberate minicircles from the concatenated kDNA network. Using these strategies, these authors showed that DNAs isolated from aliquots of *T. cruzi*-positive GEB lysates were PCR-amplified with three sets of *T. cruzi*-specific kDNA minicircle primers, yielding the 83-bp and 122-bp conserved-region fragments and the 330-bp variable-region fragments. In addition, PCR products analyzed by gel electrophoresis and/or hybridization indicated that a single *T. cruzi* cell in 20 mL of blood could be detected, increasing the sensitivity of the method. Amplification of *T. cruzi* kDNA-minicircle sequences was obtained in blood samples from five chronic-chagasic patients, even when xenodiagnosis was negative. Specific PCR amplification of minicircle sequences was also observed from heart-tissue lysates of infected mice and from abdominal contents of vector bugs. The PCR-based test proposed by Avila et al. *(39)* can therefore be useful in clinical and epidemiological studies of Chagas' disease.

The amplification of a 188-bp repetitive segment of the repetitive 195 bp-nuclear DNA sequence of *T. cruzi*, which is the most abundant sequence in this organism, was carried out by Moser at al. *(40)*. Using this strategy with DNAs from the Y, Tulahuén (Corpus Christi, and Sylvio X-10/4 isolates of *T. cruzi*, the expected 188 bp bands were evident after electrophoretic separation of the reaction products and ethidium-bromide staining. According to Moser et al. *(40)*, the results obtained suggest that 195-bp element is universally present in *T. cruzi*, since the four isolates were obtained from patients in widely separated areas (southern Brazil, Chile, TX, and northern Brazil, respectively). No amplification occurred when DNA, extracted from *Leishmania* spp or African trypanosomes, were used. In addition, as few as eight parasites in 100 μL samples of mouse blood were detected by ethidium-bromide staining of electrophoretically separated reaction products. When a radioactive probe was used to detect the amplified segment, only about 1/200 of the DNA in a single parasite was necessary for a positive identification. However, when blood samples from two persons having chronic *T. cruzi* infection were similarly tested, no amplification of the target sequence occurred *(40)*.

PCR amplification of *T. cruzi* satellite DNA was used by Dias et al. *(41)*. These authors synthesized two oligonucleotides corresponding to the most conserved region of the 195-bp repeated sequence and used these as primers for the amplification reaction. Nineteen fg of parasite DNA amplified in the presence of 15 μg of human or mouse DNA, produced a band detectable after electrophoresis in agarose gels and staining with ethidium bromide. In reconstitution experiments, one parasite in 10 mL of blood could be determined when the DNA was isolated from nuclei, after the blood was treated with NP40. Although most chronically infected mice were parasite-positive when organs or tissues were tested, all were negative when total blood was tested *(41)*.

A simple protocol for the physical cleavage of *T. cruzi* kDNA present in blood samples and its use in PCR-based diagnosis of chronic Chagas' disease was recently proposed *(42)*. In this assay, five kDNA networks (corresponding to about 10^6 minicircles) were boiled in 1 mL of blood from a healthy donor for 20 min, and after dilution the samples were amplified. The expected product of 300-bp was seen when at least 10^3 minicircles/mL were present. This procedure allows the detection of one parasite in at least 20 mL of GEB lysates. Recently, using the method proposed by Britto *(42)*, Wincker et al. *(43)* carried out a study of 100 blood samples from individuals inhabiting a single area in which Chagas' disease is endemic (Virgem da Lapa—MG, Brazil) which had given positive, negative, and indeterminant serological results. Of 86 patients previously diagnosed as chagasic by serological techniques, (IFI, IHA, and ELISA), 83 were positive (sensitivity = 96.5%), including all the xenodiagnosis-positive patients and 21 (87.5%) of 24 xenodiagnosis-negative individuals. In addition, patients with doubtful sero-

logic results were confirmed as positive by PCR. These results suggest that PCR may be a useful complement to serology in the diagnosis of Chagas' disease, and that it is the most powerful technique available for parasite detection in patients with chronic disease. This potential use of PCR would be of particular importance in cases in which serology cannot serve as a good indicator of the presence or absence of *T. cruzi*, for example, in patients undergoing specific treatment *(44)*. The results obtained by these approaches show that additional work needs to be done with PCR-based detection assays to define their sensitivities and specificities. It is important to emphasize that the detection of parasite DNA by PCR indicates active infection, in contrast to the diagnostic methods based on detection of antibodies.

CONCLUSION

The use of recombinant antigens and synthetic peptides as reagents for the serological diagnosis of chronic Chagas' disease, and the identification of parasites in the blood by PCR techniques, have proven to be major advances. However, studies performed thus far have involved a limited number of well-characterized patients. Further studies are necessary to achieve the ideal test, which should be cheap, quick, easily performed, reliable, and highly specific and sensitive.

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