

## CHAGAS' DISEASE DIAGNOSIS: COMPARATIVE ANALYSIS OF PARASITOLOGIC, MOLECULAR, AND SEROLOGIC METHODS

MONICA L. GOMES, LUCIA M. C. GALVAO, ANDREA M. MACEDO, SERGIO D. J. PENA, AND EGLER CHIARI

Departamento de Parasitologia e Departamento de Bioquímica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; Departamento de Análises Clínicas, Universidade Estadual de Maringá, Maringá, Brazil

**Abstract.** During the course of chronic chagasic infection, low parasitemia levels prevent parasite detection by current techniques such as hemoculture and xenodiagnosis. Since serologic tests have sensitivity but lack specificity, molecular assays based on the polymerase chain reaction (PCR) have been proposed as alternative tools for parasite detection in individuals with chronic Chagas' disease. A variable degree of PCR efficiency has been reported in the literature and illustrates the need for further evaluation of large numbers of chagasic patients. In this study, we compared an optimized PCR technique with hemoculture and complement-mediated lysis (CoML) in 113 individuals from or living in endemic areas of Brazil who had conventional serologic results that were either positive, negative, or inconclusive. The PCR amplification yielded positive results in 83.5% (66 of 79) of individuals with positive serology, 47.6% (10 of 21) with negative serology, and 46.2% (6 of 13) with inconclusive serology. Of 10 patients with negative serology and positive PCR result, eight (80%) had positive CoML, indicating that they could have been chagasic but were not mounting immune responses. The PCR results were also positive for all individuals who had positive hemoculture, for 37 individuals with negative hemoculture and positive serology, and for two of six individuals with inconclusive serology and negative hemoculture. Thirteen individuals living in nonendemic areas who had negative serology were used as a negative control group: 100% had negative PCR results. Our results show that the optimized PCR protocol used here was very sensitive in detecting the presence of *Trypanosoma cruzi* in chronic chagasic patients. The PCR and CoML results were well correlated in all of the groups studied, which suggests that our PCR protocol may be effective in the evaluation of cure in patients who receive anti-parasite treatment.

Different approaches have been used in the diagnosis of chronic Chagas' disease. Serologic tests are used to detect antibodies against *Trypanosoma cruzi* and not the presence of the parasite itself. These tests have high sensitivity but lack specificity because of antigenic cross-reactivity with parasites such as *Leishmania* sp. and *T. rangeli*.<sup>1,2</sup> Parasitologic tests such as hemoculture or xenodiagnosis have proven to be highly specific, but the sensitivity of these techniques is low. Recently, molecular assays such as the polymerase chain reaction (PCR), which amplify certain repetitive sequences of trypanosome kinetoplast DNA (kDNA) have been proposed as a good alternative tools for detection of *T. cruzi* in human blood.<sup>3-5</sup> The ≈330-basepair (bp) fragment of the kinetoplast minicircles is normally used as a target for amplification.

The PCR assay has shown a variable degree of efficiency. Initial sensitivity reports ranged from 96% to 100% compared with serologic diagnosis.<sup>3,4</sup> A lower sensitivity level was observed by Britto and others<sup>6</sup> and Junqueira and others.<sup>7</sup> These inconsistencies illustrate the need for additional evaluation of large numbers of chagasic individuals from different endemic regions in Brazil due to extensive variations in the incidence and clinical manifestations of Chagas' disease in this country.

A new technique to verify cure in chagasic patients who received specific treatment is needed. Conventional serologic tests such as the indirect immunofluorescence (IIF) test, the indirect hemagglutination test, and the ELISA are ineffective because they are persistently positive in most treated patients even when parasitologic test results are negative.<sup>8</sup> Since current parasitologic methods have low sensitivity, a negative result does not necessarily mean that the individual is free of infection. Krettli and Brener proposed complement-mediated lysis (CoML), which detects lytic antibodies, as an alternative method.<sup>9</sup> The presence of these antibodies indicates an active infection and the absence indicates cure.<sup>10</sup>

However, CoML is labor-intensive and time-consuming because it requires living trypomastigotes, human serum as a complement source, and counting numerous parasites by light microscopy.

In this study, we compared an optimized PCR technique with hemoculture and CoML in detecting *T. cruzi* infection in individuals from different endemic and nonendemic regions of Brazil who had conventional serologic results that were either positive, negative, or inconclusive.

### MATERIALS AND METHODS

**Subjects.** Data were collected from 113 individuals who had lived in various Chagas' disease endemic areas of Brazil and who were living in southern Brazil at the time of the study. Thirteen individuals from nonendemic regions were used as a negative control group to verify the specificity of the PCR protocol. The epidemiologic and serologic characteristics of the study populations are shown in Table 1. The clinical state of the subjects was determined by conventional electrocardiography and thoracic radiography. Individuals were submitted to serologic and parasitologic tests for Chagas' disease and classified into four groups: 1) positive serology and hemoculture, 2) positive serology and negative hemoculture, 3) inconclusive serology, and 4) negative serology. The study protocol was approved by the Institutional Review Committee of the Universidade Estadual de Maringá. All study participants provided informed consent.

**Serologic tests.** Serum was prepared from the blood of each individual and conventional serology was performed by IIF (positive ≥ 1:40 dilution; Biolab, Jacarepagua, Rio de Janeiro, Brazil) and ELISA (Abbott Laboratories, Sao Paulo, Brazil) in accordance with the manufacturers' instructions. An individual was considered positive when the two test results were positive, negative when both results were negative, and inconclusive when only one result was positive.

TABLE 1  
Epidemiologic and serologic characteristics of the study population\*

Serology	No.	Age range, years (mean)	Sex M/F	From or living in endemic area (%)	No blood transfusion (%)	Living in house where a triatomine bug was present (%)
P†	79	11–76 (46.3)	34/45	100.0	83.8	65.7
N†	21	31–69 (50.2)	8/13	100.0	85.7	46.1
I†	13	19–62 (44.3)	4/9	100.0	75.0	37.5
N‡	13	ND	ND	0	100.0	0

\* P = positive; N = negative; I = inconclusive; ND = not determined.

† There were no statistically significant differences in epidemiologic characteristics ( $P < 0.05$ ) between groups of individuals from different endemic areas who were living in north-west Parana State. Age was analyzed using the Student's *t*-test and the variables sex, history of blood transfusion, and living in a house where a triatomine bug was present were analyzed using the chi-square test.

‡ Control group.

Lytic antibodies were detected by CoML.<sup>11</sup> Trypomastigotes ( $6 \times 10^6$ /ml) were incubated with human complement (HuC) at 37°C for 30 min and counted to ensure total resistance to complement lysis in the absence of immune serum. Fifty microliters of a suspension of the trypomastigotes and 50  $\mu$ l of two-fold and four-fold diluted test serum were incubated at 37°C for 30 min and placed on ice. Fifty microliters of HuC were added to 50  $\mu$ l of each sample and the parasites were counted using an hemocytometer. The tubes were incubated at 37°C for 45 min, placed on ice, and the parasites were recounted. For each test, a serum from a healthy control and a positive control serum were included and all readings were double-blind. The sera were considered positive when lysis was superior 20%.

**Hemoculture.** Total blood (30 ml) was collected into tubes containing heparin from each individual with positive or inconclusive conventional serology and centrifuged at 4°C to harvest the plasma. The packed cells were washed twice by centrifugation at 4°C in liver infusion tryptose (LIT) medium,<sup>12</sup> distributed among six tubes containing 3 ml of LIT medium, and incubated at 28°C.<sup>13</sup> All tubes were mixed gently once a week and examined monthly for up to 120 days.

**Preparation of DNA for the PCR.** Blood (15 ml) was collected from subjects and immediately mixed with an equal volume of a solution of 6 M guanidine hydrochloride (Sigma Chemical Company, St. Louis, MO) and 0.2M EDTA.<sup>14</sup> The samples were heated for 15 min in boiling water to shear the DNA molecules.<sup>15</sup> After cooling, 200  $\mu$ l were taken from each subject's blood lysate and DNA extraction was performed exactly as described by Gomes and others.<sup>16</sup>

**Polymerase chain reaction conditions.** To detect the *T. cruzi* kDNA  $\approx$ 330-bp fragment, PCR amplification was done in a 20- $\mu$ l reaction mixture containing 10 mM Tris-HCl, pH 9.0, 75 mM KCl, 3.5 mM MgCl<sub>2</sub>, 20 pmoles of 121 and 122 primers<sup>3</sup> (Operon Technology, Inc., Alameda, CA), 200  $\mu$ M dNTPs, 1.0 unit of *Taq* DNA polymerase (Promega, Madison, WI), and 2  $\mu$ l of DNA template. Mineral oil (30  $\mu$ l) (Sigma Chemical Company) was added to prevent evaporation. Reactions were performed using an MJ Research (Watertown, MA) programmable thermal cycler (PTC-150).

The cycle steps and analysis of PCR products were done exactly as reported by Gomes and others.<sup>16</sup> The PCR controls were added to each series of samples to verify that carryover DNA contamination did not occur.

## RESULTS

**Detection of the 330-bp fragment of *T. cruzi* kDNA in human blood by PCR.** An optimized PCR method was used on DNA isolated from the blood of infected and uninfected individuals from endemic and nonendemic areas. This PCR protocol has a sensitivity of 0.1% fg of kDNA after hybridization with a specific probe, which corresponds to one intact parasite or 0.01% of the *T. cruzi* DNA fragment circulating in the blood of an infected host. Representative samples for each study group are shown in Figure 1. The 330-bp and 750-bp specific products were detected in subjects who had positive serology and hemoculture results and in those with positive serology and negative hemoculture results. An additional amplified DNA fragment of 199 bp derived from human DNA<sup>16</sup> was also found in positive and negative *T. cruzi* samples and was used as an internal control of PCR inhibition.

Samples in which the DNA concentration was very low are shown in Figure 2. For these samples, we identified PCR-amplified DNA using slot-blot hybridization with an alkaline phosphatase-labeled probe<sup>16</sup> (Figure 3).

**Comparison of the PCR with CoML in individuals with positive, negative, or inconclusive conventional serologic results.** The PCR diagnosis was performed on 126 samples from individuals from endemic (113) and nonendemic (13) areas. Of those individuals from endemic areas who had positive serology, 83.5% (66 of 79) also had positive kDNA amplification. In addition, 47.6% (10 of 21) of those with negative serology and 46.2% (6 of 13) with inconclusive serology had positive PCR results (Table 2). The CoML results were similar: 84.8% (67 of 79), 52.4% (11 of 21), and 38.5% (5 of 13) of the individuals from endemic areas with positive, negative, or inconclusive serology, respectively, had positive CoML results (Table 2). However, of those individuals from nonendemic areas who had negative serology, none had positive PCR or CoML results.

**Comparison of PCR diagnosis with hemoculture.** Of the 92 individuals who had positive or inconclusive serology, 85 were also examined by hemoculture. Only 36.5% (29) of the individuals who had positive serology had positive hemoculture results while the results of the PCR were positive for all individuals with positive hemoculture results and for 46.8% (37) individuals with negative hemoculture results. The results of the PCR were also positive for two of six subjects who had inconclusive serology and negative hemoculture results (Table 3).

## DISCUSSION

In the present study, an optimized PCR technique detected *T. cruzi* kDNA in the blood of 83.5% of the individuals with positive serology from various areas endemic for Chagas' disease. Previous studies reported PCR sensitivities of 96.5% and 100% in blood of chronic chagasic patients from Virgem da Lapa, Minas Gerais, Brazil.<sup>3,4</sup> The chagasic patients living

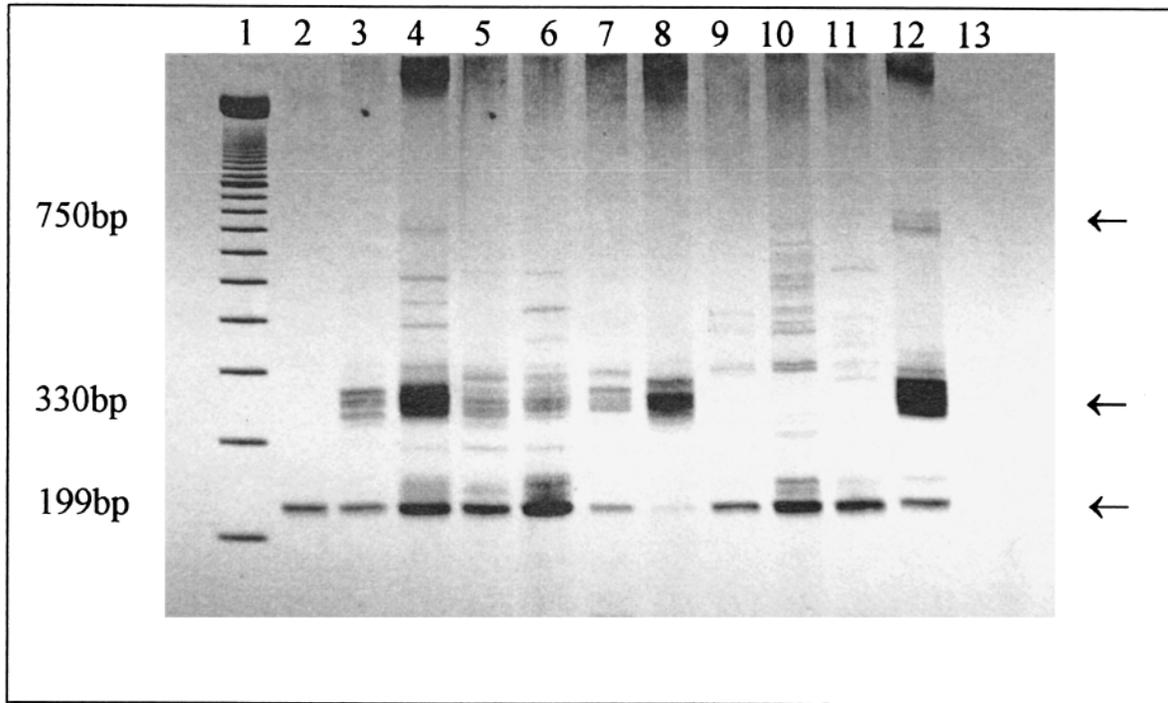


FIGURE 1. Representative polyacrylamide gel electrophoresis of blood samples from different groups analyzed by the polymerase chain reaction (PCR). Lane 1, molecular weight marker (100-basepair [bp] ladder; GIBCO-BRL Life Technologies, Gaithersburg, MD); lane 2, DNA extracted from the blood of a nonchagasic individual (negative control); lanes 3–5, DNA from the blood of individuals with positive serology, hemoculture, and complement-mediated lysis (CoML) results; lanes 6–8, DNA from the blood of individuals with positive serology and CoML and negative hemoculture; lanes 9–11, DNA from the blood of individuals with negative serology; lane 12, DNA from the blood of a chronic chagasic patient (positive control); lane 13, no DNA in the reaction mixture for the PCR amplification. The **arrows** indicate *Trypanosoma cruzi*-specific products of 330 bp, 750 bp, and 199 bp in human blood from negative and positive samples for *T. cruzi*.

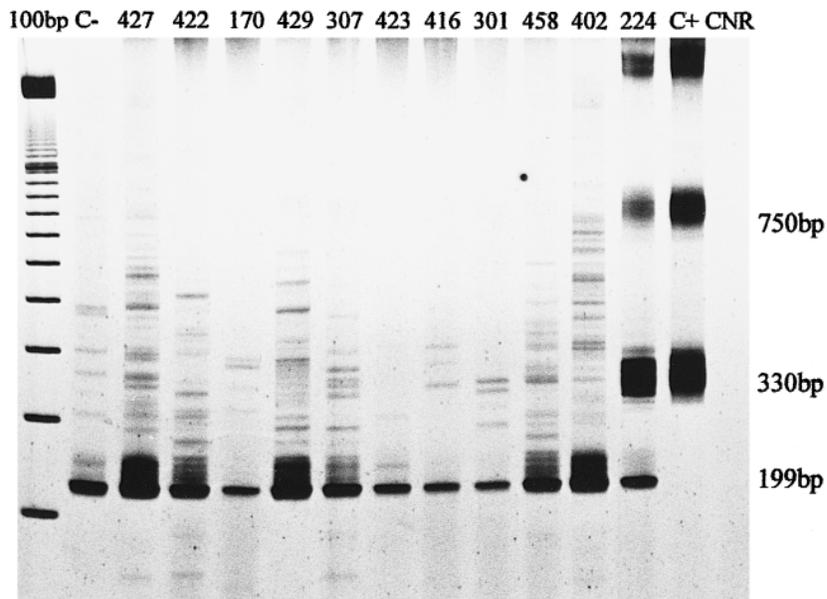


FIGURE 2. Polyacrylamide gel electrophoresis (silver staining) of polymerase chain reaction (PCR) products amplified from DNA extracted from the blood of subjects 427, 458, and 402 with positive serology, hemoculture, and complement-mediated lysis (CoML); of subjects 170, 429, 423, 416, 301, and 224 with positive serology and CoML and negative hemoculture; and of subjects 422 and 307 with positive serology, negative hemoculture, and negative CoML. C- = DNA from the blood of a nonchagasic individual; C+ = 10 pg of *Trypanosoma cruzi* total DNA; CNR = no DNA in the reaction mixture for the PCR amplification. bp = basepairs.

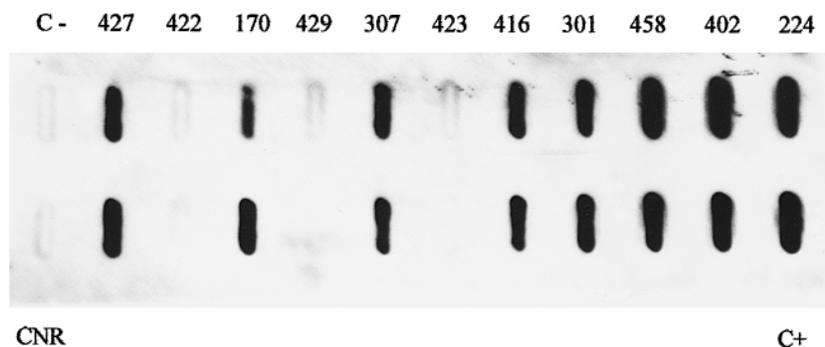


FIGURE 3. Slot-blot hybridization with alkaline phosphatase-labeled oligonucleotide probe of the same polymerase chain reaction (PCR) products shown in Figure 2. Duplicate samples are shown. C- = DNA from the blood of a nonchagasic individual; CNR = no DNA in the reaction mixture for the PCR amplification; C+ = 10  $\mu$ g of *Trypanosoma cruzi* total DNA.

in this region had high parasitemia levels based on positive xenodiagnosis results compared with individuals from other endemic areas.<sup>17</sup> This phenomenon could explain the PCR results observed in patients from Virgem da Lapa. Compared with conventional serologic tests, we found a higher PCR sensitivity than those reported by Britto and others<sup>6</sup> and Junqueira and others<sup>7</sup> (45% and 60%, respectively), who used the protocol reported by Wincker and others.<sup>3</sup>

The PCR results were also positive for 46.2% (6 of 13) of individuals with inconclusive serology and for 47.6% (10 of 21) of individuals with negative serology who were living in endemic areas (Table 2). We thoroughly investigated possible reasons for this unexpectedly high percentage of individuals with negative serology and positive PCR results. Contamination during the PCR procedure is unlikely since we performed several DNA extractions and PCR experiments at different times, using different blood samples and separate laboratory areas. The specificity of the PCR products was also investigated by internal probe hybridization. Also, the negative control group as well as our negative internal controls were consistently negative and they were processed simultaneously with the study samples. This is not the first time that PCR results were positive in individuals with negative serology. Avila and others reported positive PCR results in one patient with negative serology with cardiomyopathy characteristic of chronic Chagas' disease, and in 66.7% (2 of 3) of presumed nonchagasic subjects from an area endemic for Chagas' disease.<sup>4</sup> Individuals with negative serology in the present study did not exhibit clinical manifestations characteristic of Chagas' disease, but a high percentage of them were from endemic areas and lived in houses where triatomine bugs were present (Table 1). Since

the CoML was also positive for most (8 of 10) of these individuals, it is possible that they were infected. It is known that some chronic chagasic patients with positive xenodiagnosis results consistently have negative serologic results throughout their lives.<sup>18,19</sup> We believe that the number of reports of these cases is not higher because parasitologic tests are usually not performed in individuals with negative serology. Our results, as well as others reported in the literature,<sup>19-21</sup> suggest that conventional serologic tests may be unable to detect infection in some chronic chagasic individuals. This might be due to specific humoral depression in these patients<sup>19-21</sup> or the inability of these tests to detect low levels of antibodies to *T. cruzi*. In addition to our findings, discordant conventional serologic test results for Chagas' disease diagnosis in blood donor screening have been reported by other researchers.<sup>22-26</sup>

This study was a comparative analysis of PCR and CoML methods in groups of individuals with positive, negative, or inconclusive conventional (IIF, ELISA) serologic results. Of the 113 subjects, 87 (77%) had concordant results for both tests (Table 2). The CoML has been used to assess cure after specific treatment of Chagas' disease, but the PCR may be a more useful tool because it has the advantage of detecting the presence or absence of the parasite itself. The first attempt to evaluate the performance of the PCR in individuals who received treatment but were still serologically positive was done by Britto and others.<sup>5</sup> These investigators observed positive PCR results in 90% of untreated individuals and in 28% of patients who received treatment with benznidazole. However, these data were collected at short-term, post-treatment follow-up, and the PCR was not performed prior to treatment.

The PCR and hemoculture methods were performed on 85 individuals. The hemoculture results were positive for 36.5% (29) of the individuals with positive serology, while the PCR detected infection in 83.5% (66). The results of the PCR were positive in two of six individuals who had inconclusive serology (Table 3). Taken together with the results of previous studies,<sup>3-7</sup> our results showed a consistent increase in PCR sensitivity compared with hemoculture in the same group of individuals.

The results of the current study suggest a need for future analysis of the two approaches. Specifically, two issues need

TABLE 2

Comparison of the polymerase chain reaction (PCR) with complement-mediated lysis (CoML) of individuals from various endemic areas of Brazil living in northwest Parana State

Serology	No.	PCR		CoML	
		+	-	+	-
Positive	79	66	13	67	12
Negative	21	10	11	11	10
Inconclusive	13	6	7	5	8
Total	113	82	31	83	30

TABLE 3  
Comparison of results for blood samples analyzed by hemoculture and the polymerase chain reaction

Sample type	No. of samples	PCR results	
		+	-
Positive serology and hemoculture	29	29	0
Positive serology, negative hemoculture	50	37	13
Inconclusive* serology, negative hemoculture	6	2	4

\* Hemoculture was performed only on a subset (6 of 13) of these individuals.

to be addressed. The first is to verify the usefulness of the PCR as a tool to assess cure after specific chemotherapy. Experiments are in progress in our laboratory to ascertain the efficiency of our PCR protocol in monitoring cure after specific treatment. The second question involves positive PCR results observed for individuals with negative serologic results. This could be clarified through extensive analysis of the PCR and other Chagas' disease diagnostic methods in blood donor screening.

Acknowledgment: We thank Dawn Kelly for assistance with language revision.

Financial support: This work was supported by grants from the Fundação de Amparo a Pesquisa de Minas Gerais, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Financiadora de Estudos e Projetos, 41/96/0894/00 (Pronex), and a Coordenação de Aperfeiçoamento do Pessoal de Ensino Superior-Plano Institucional de Capacitação Docente fellowship.

Authors' addresses: Mônica L. Gomes, Departamento de Análises Clínicas, Universidade Estadual de Maringá, Av. Colombo, 5790 87020-900 Maringá, Parana, Brazil. Lucia M. C. Galvão and Egler Chiari, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (ICB/UFMG), Av. Antonio Carlos, 6627 Caixa Postal 486, Pampulha 31270-901, Belo Horizonte, Minas Gerais, Brazil. Andréa M. Macedo and Sérgio D.J. Pena, Departamento de Bioquímica e Imunologia, ICB/UFMG, Av. Antonio Carlos, 6627 Pampulha 31270-910, Belo Horizonte, Minas Gerais, Brazil.

#### REFERENCES

- Schmunis GA, 1991. *Trypanosoma cruzi*, the etiological agent of Chagas disease: status in blood supply in endemic countries. *Transfusion* 31: 547-557.
- Saldana A, Sousa OE, 1996. *Trypanosoma rangeli*: epimastigote immunogenicity and cross-reaction with *Trypanosoma cruzi*. *J Parasitol* 82: 363-366.
- Wincker P, Britto C, Borges-Pereira J, Cardoso MA, Oelemann W, Morel CM, 1994. 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.
- Avila H, Borges-Pereira J, Thiemann O, de Paiva E, Degraive W, Morel CM, Simpson L, 1993. Detection of *Trypanosoma cruzi* in blood specimens of chronic chagasic patients by polymerase chain reaction amplification of kinetoplast minicircle DNA: comparison with serology and xenodiagnosis. *J Clin Microbiol* 31: 2421-2426.
- Britto C, Cardoso MA, Vanni CMM, Hasslocher-Moreno A, Xavier S, Oelemann W, Santoro A, Pimimez C, Morel CM, Wincker P, 1995. Polymerase chain reaction detection of *Trypanosoma cruzi* in human blood samples as a tool for diagnosis and treatment evolution. *Parasitology* 110: 241-247.
- Britto C, Cardoso MA, Ravel C, Santoro A, Borges-Pereira J, Coura JR, Morel CM, Wincker P, 1995. *Trypanosoma cruzi*: parasite detection and strain discrimination in chronic chagasic patients from northeastern Brazil using amplification of kinetoplast DNA and nonradioactive hybridization. *Exp Parasitol* 81: 462-471.
- Junqueira ACV, Chiari E, Wincker P, 1996. Comparison of the polymerase chain reaction with two classical parasitological methods for the diagnosis of Chagas disease in an endemic region of north-eastern Brazil. *Trans R Soc Trop Med Hyg* 90: 129-132.
- Krettli AU, Cangado JR, Brener Z, 1984. Criterion of cure of human Chagas' disease after specific chemotherapy: recent advances. *Mem Inst Oswaldo Cruz* 79: 157-164.
- Krettli AU, Brener Z, 1982. Resistance against *Trypanosoma cruzi* associated to anti-living trypomastigote antibodies. *J Immunol* 129: 2009-2012.
- Krettli AU, Cancado JR, Brener Z, 1982. Effect of specific chemotherapy on the levels of lytic antibodies in Chagas disease. *Trans R Soc Trop Med Hyg* 76: 334-340.
- Galvao LMC, Nunes RMB, Cancado JR, Brener Z, Kretth AU, 1993. Lytic antibody titre as a means of assessing cure after treatment of Chagas disease: a 10 year follow-up study. *Trans R Soc Trop Med Hyg* 87: 220-223.
- Camargo EP, 1964. Growth and differentiation in *Trypanosoma cruzi*. Origin of metacyclic trypanosomes in liquid media. *Rev Inst Med Trop Sao Paulo* 6: 93-100.
- Chiari E, Dias JCP, Lana M, Cifari CA, 1989. Hemocultures for the parasitological diagnosis of human chronic Chagas disease. *Rev Soc Bras Med Trop* 22: 19-23.
- Avila AH, Sigman DS, Cohen LM, Millikan RC, Simpson L, 1991. Polymerase chain reaction amplification of *Trypanosoma cruzi* kinetoplast minicircle DNA isolated from whole blood lysates: diagnosis of chronic Chagas disease. *Mol Biochem Parasitol* 48: 211-222.
- Britto C, Cardoso MA, Wincker P, Morel CK 1993. A simple protocol for the physical cleavage of *Trypanosoma cruzi* kinetoplast DNA present in blood samples and its use in polymerase chain reaction (PCR)-based diagnosis of chronic Chagas disease. *Mem Inst Oswaldo Cruz* 88: 171-172.
- Gomes ML, Macedo AK Vago AR, Pena SDJ, Galvao LMC, Chiari E, 1998. *Trypanosoma cruzi*: optimization of polymerase chain reaction for detection in human blood. *Exp Parasitol* 88: 28-33.
- Borges-Pereira J, Junqueira AC, Santos LC, Castro JAF, Araujo EB, Coura JR, 1996. Xenodiagnóstico na doença de Chagas crônica. I—Sensibilidade de *Panstrongylus megistus* e *Triatoma infestans*. *Rev Soc Bras Med Trop* 29: 341-347.
- Camargo ME, 1992. Serological diagnosis: an appraisal of Chagas disease serodiagnosis. Wendel S, Brener Z, Camargo ME, Rassi A, eds. *Chagas Disease (American Trypanosomiasis): Its Impact on Transfusion and Clinical Medicine*. Sao Paulo: Sociedade Brasileira de Hematologia e Hemoterapia, 165-178.
- Luquetti AO, 1987. Megaesôfago e anticorpos anti-*Trypanosoma cruzi*. *Rev Goiana Med* 33: 1-16.
- Breniere SF, Carrasco R, Antezana G, Desjeux P, Tibayrenc M, 1989. Association between *Trypanosoma cruzi* zymodemes and specific humoral depression in chronic chagasic patients. *Trans R Soc Trop Med Hyg* 83: 517.
- Breniere SF, Poch O, Selaes H, Tibayrenc M, Lemesre J, Antezana G, Desjeux P, 1984. Special humoral depression in chronic patients infected by *Trypanosoma cruzi*. *Rev Inst Med Trop Sao Paulo* 26: 245-258.
- Pless M, Juranek D, Kozarsky P, Steurer F, Tapia G, Bennudez

- H, 1992. The epidemiology of Chagas' disease in a hyperendemic area of Cochabamba, Bolivia: a clinical study including electrocardiography, seroreactivity to *Trypanosoma cruzi*, xenodiagnosis, and domiciliary triatomine distribution. *Am J Trop Med Hyg* 47: 539–546.
- 23 Andrade ALSS, Martelli CMT, Luquetti AO, Oliveira OS, Almeida e Silva S, Zicker F, 1992. Serologic screening for *Trypanosoma cruzi* among blood donors in Central Brazil. *Bull Pan Am Health Organ* 26: 157–164.
- 24 Lorca HM, Child GR, Garcia CA, Silva GM, Osorio SJ, Atias MA, 1992. Evaluacion de reactivos comerciales empleados en el diagnostics de la enfermedad de Chagas en bancos de sangre de Chile. 1. Seleccion de reactivos. *Rev Med Chil* 120: 420–426.
- 25 Carvalho MR, Kreiger MA, Almeida E, Oelemann W, Shikanai-Yasuda MA, Ferreira AW, Perreira JB, Saez-Alquezar A, Dorhiac-Llaer PE, Chamone DF, Goldenberg S, 1993. Chagas disease diagnosis evaluation of several tests in blood screening. *Transfusion* 33: 830–834.
- 26 Salles NA, Sabino EC, Cliquet MG, Eluf-Neto J, Mayer A, Almeida-Neto C, Mendonca MC, Dorliach-Llacer P, Chamone DF, Saez-Alquezar A, 1996. Risk of exposure to Chagas disease among seroreactive Brazilian blood donors. *Transfusion* 33: 969–973.