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Processo Nº CBB 2319/97

CARACTERIZAÇÃO MOLECULAR DO Trypanosoma cruzi NO SANGUE E TECIDOS DE PACIENTES CHAGÁSICOS PORTADORES DE MEGAESÔFAGO

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1. Resumo da Pesquisa

A persistência tecidual e sanguínea do Trypanosoma cruzi na forma digestiva da doença de Chagas crônica foi avaliada em 52 pacientes portadores de megaesófago chagásico submetidos a cirurgia terapêutica do mesmo, por meio de técnicas parasitológicas (xenodiagnóstico e/ou hemocultura), histopatológicas (colorações pela hematoxilina-eosina e peroxidase-anti-peroxidase (PAP) e moleculares [reação em cadeia da polimerase (PCR) seguida de hibridização em Slot-dot].

A presença do T. cruzi e ou de seus fragmentos genômicos foi demonstrada aleatoriamente ou seja, sem a previa determinação dos focos inflamatórios, em 76,9% dos esôfagos estudados e no sangue destes pacientes em 90,4% por meio da PCR e em 73,1% pelos métodos parasitológicos. O processo inflamatório foi encontrado em 50% dos tecidos dos quais 80,8% associados com o parasita. O T. cruzi também foi detectado não associado com alterações inflamatórias, sendo observada a presença destas na ausência do T. cruzi. Nos tecidos esofágicos dos pacientes com megaesófago grau IV observamos um maior parasitismo e intensidade dos processos inflamatórios. Estes dados demonstram que na forma digestiva da doença de Chagas, especificamente nos casos de megaesófago, a presença frequente do T. cruzi tanto no sangue como nos tecidos deve de algum modo participar dos mecanismos patogênicos desenvolvidos nesta forma clínica da infecção chagásica humana.

A técnica de LSSP-PCR foi realizada em 22 dos tecidos esofágicos PCR positivos, pois os demais tecidos apesar de positivos apresentaram amplificações com quantidade de DNA insuficiente para a realização da técnica.

Os perfis de assinatura gênica do DNA do cinetoplasto (kDNA) do T. cruzi foram muito heterogêneos e característicos para cada paciente, não sendo possível determinar um padrão definido de sub-populações do parasita envolvidas na gênese do megaesófago chagásico. Ao agruparmos os perfis de assinatura gênica do T. cruzi no tecido esofágico de acordo com as manifestações clínicas dos pacientes verificamos que esta variabilidade também ocorre entre os pacientes de uma mesma forma clínica inclusive entre aqueles que apresentavam apenas megaesófago ou a forma digestiva, bem marcada ou seja megaesófago e megacólon. O grau do megaesófago e a associação com as formas cardíacas, que poderiam refletir uma maior capacidade de lesão das cepas do parasita, também não permitiram agrupar padrões semelhantes de subpopulações do T. cruzi, sendo observada uma heterogeneidade das mesmas tanto nos pacientes com megaesófagos pouco dilatados (grau II) como nos avançados (grau IV).

A diversidade de perfis genéticos encontrada reflete a variabilidade do kDNA presente nos minicírculos do parasita, sugerindo que, provavelmente, mais de uma subpopulação do T. cruzi deve estar presente nesta forma clínica.

Comparamos os perfis de low-stringency single specific primer PCR (LSSP-PCR) obtidos do tecido, sangue e isolados (hemocultura, xenocultura e ou fezes de triatomíneos) de 10 pacientes. Os perfis de bandas gerados pelas subpopulações do T. cruzi presentes nos isolados a partir da hemocultura, xenocultura e fezes de triatomíneos na reação de LSSP-PCR mostraram uma identidade entre elas, levando a concluir que tanto no vetor como no meio de cultura artificial (LIT) desenvolveram as mesmas subpopulações do parasita.

As sub-populações do T. cruzi presentes nas cepas provenientes de pacientes com diferentes formas clínicas da infecção chagásica, foram isoladas por hemocultura e ou xenodiagnóstico. Amastra de 24 pacientes representantes das seguintes formas clínicas: digestiva (2 pacientes com megacólon, 7 com megaesófago), cardio-digestiva (4 pacientes associados megaesófago e 4 com megaesófago e megacólon) e cardíaca (7 pacientes). Nestas amostras comparamos os perfis das assinaturas gênicas do parasita amplificadas pela reação de LSSP-PCR incluindo também algumas amostras de
xenodiagnóstico e hemocultura de um mesmo paciente como duplicatas, uma vez que ambas apresentam o mesmo perfil de sub-populações.

De modo semelhante ao que aconteceu nos tecidos, novamente observamos uma variabilidade do kDNA do *T. cruzi* presente entre os isolados responsáveis pelas diferentes formas clínicas (formas digestiva, cardíaca e cardiodigestiva) assim como entre aquelas sub-populações responsáveis por uma mesma forma clínica da doença de Chagas humana. Este dado vem reforçar o achado anterior no qual estudando as sub-populações do *T. cruzi* diretamente envolvidas na gênese das lesões teciduais do megaesôfago, também detectamos padrões heterogêneos característicos para cada paciente.

O diagnóstico da infecção chagásica em pacientes co-infetados pelo HIV foi demonstrado com eficiência e rapidez pela PCR, sugerindo ter essa técnica um grande potencial para confirmar a presença ou ausência do *T. cruzi* no líquor e controle terapêutico nos casos de invasão do sistema nervoso central. As populações de parasitas obtidas do sangue e líquor foram caracterizadas pela LSSP-PCR e PCR usando sequências do gene 24Sα do RNA ribossomal (rRNA) e em ambas populações mostraram homogeneidade de k-DNA e a presença da linhagem 1 do *T. cruzi II*.

2. Palavras-chaves

*Trypanosoma cruzi*; Doença de Chagas; Megaesôfago chagásico; *Polymerase chain reaction* (PCR); Peroxidase-anti-peroxidase (PAP); Testes parasitológicos; *Low-stringency single specific primer PCR* (LSSP-PCR); Síndrome de imunodeficiência adquirida (SIDA); RNA ribossomal (rRNA)

3. Data do Auxílio

Início: 27/07/1998

Término: 27/07/2000
4. Anexos

4.1 Exame de “Qualify”

Cópia da Ata de Defesa de “Qualify” de Eliane Lages Silva Nº Registro 23/99, aluna de doutorado do Programa de Pós-graduação em Parasitologia e nossa orientada.

Gostaria de informar a V. Sa. que a tese está em fase de redação com defesa prevista para julho/2001.

4.2 Trabalhos Aceitos para Publicação em 2001

4.2.1 Carta e cópia do 1º trabalho

_American Journal of Tropical Medicine and Hygiene_

4.2.2 Carta e cópia do 2º trabalho

_Clinical Infectious Diseases_

4.3 Cópia das apresentações orais e resumos de trabalhos em Congressos e Reuniões Científicas

4.3.1 Mesas redonda: 02

4.3.2 Posters: 02
ATA DE DEFESA DO EXAME DE “QUALIFY” DE ELIANE LAGES SILVA,
Orientador: Prof. Egler Chiari.

Às 10:00 horas do dia 09 do mês de dezembro de 1999, reuniu-se no Instituto de Ciências Biológicas da UFMG, no Departamento de Parasitologia, a Comissão Examinadora do Exame de “Qualify”, indicada pelo Colegiado do Programa, para julgar o trabalho intitulado: “Caracterização molecular do Trypanosoma cruzi no sangue e tecidos de pacientes chagásicos portadores de megaesôfago”, requisito para defesa final da tese. Abrindo a sessão, o(a) Presidente da Comissão, Prof.(a) Egler Chiari, passou a palavra ao(a) candidato(a) para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva apresentação do(a) candidato(a). Logo após, a Comissão se reuniu, sem a presença do(a) candidato(a), para julgamento e expedição do resultado final.

O trabalho do(a) candidato(a) foi considerado **Aprovado**.

Nada mais havendo a tratar o(a) Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 09 de dezembro de 1999.

**Dr. Egler Chiari**
(Orientador)

**Dra. Élida Mara Leite Rabelo**

**Dr. Evaldo Nascimento**
THE RELATIONSHIP BETWEEN *Trypanosoma cruzi* AND HUMAN CHAGASIC MEGAESOPHAGUS

HUMAN CHAGASIC MEGAESOPHAGUS: BLOOD AND TISSUE PARASITISM

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Abstract. The persistence of *Trypanosoma cruzi* in tissue and blood of 52 patients in the digestive form of chronic Chagas' disease was studied. These patients had chagasic megaesophagus and underwent corrective surgery. Parasitologic (xenodiagnosis and/or hemoculture), histopathologic (hematoxylin-eosin and peroxidase-anti-peroxidase staining) and molecular (polymerase chain reaction [PCR] followed by slot-blot hybridization) tests were used in the analysis. The presence of *T. cruzi*, its genomic fragments and/or its antigens could be detected in 98% (51/52) of the patients. The parasite was randomly identified in 76.9% of esophageal tissues and in 90.4% by PCR and in 73.1% by parasitologic methods from the blood. Fifty percent (26/52) of tissue samples had inflammation, 80.8% of which was associated with the parasite. *T. cruzi* was also identified unassociated with inflammatory alterations. Higher tissue parasitism and intense inflammatory processes were observed in esophageal tissue from patients with grade IV megaesophagus. These data demonstrate that in the digestive form of Chagas' disease, particularly in cases of megaesophagus, *T. cruzi* is frequently found, both in blood and tissues, and may contribute to the pathogenic mechanisms involved.

Although Chagas' disease was discovered nine decades ago, its pathogenesis and the mechanisms of parasite-host relationship are still not well understood. This infection has a variable clinical course, most of patients remain free of alterations in the indeterminate phase, others can develop to severe chronic disease, with cardiac or gastrointestinal involvement. The prevalence of digestive manifestations during the chronic phase of the disease varies considerably in different endemic areas. The digestive form seems to be absent from certain countries like Panama and Venezuela,\(^1,2\) while in central Brazil, 2-8.8% of patients have megaesophagus.\(^3,6\)
The pathogenesis of chagasic megaesophagus may be related to host characteristics as well as to different strains of the parasite *Trypanosoma cruzi*. One of the most important factors that determines both symptoms and complications of this clinical form is the denervation of the smooth muscle of the esophagus causing several morphological and motor changes, according to which megaesophagi are classified by grades I to IV. Until a short time ago microscopic techniques were unable to disclose parasites in the affected tissues, leading many authors to invoke auto-immune mechanisms as responsible for the cellular lesions.

More recently, new highly sensitive techniques such as immunohistochemistry and protocols based on the polymerase chain reaction (PCR) have consistently shown the presence of the parasites in diseased organs and their absence from non-affected tissues. This, together with an increase in efficiency in detection of *Trypanosoma cruzi* in blood by serial hemocultures and by PCR has provided convincing evidence that presence of parasites in tissues is associated with the pathogenic process.

In the present study chagasic megaesophagus was evaluated using parasitologic, clinical and histopathologic approaches. Specific PCR, followed by hybridization as well as peroxidase-anti-peroxidase (PAP) and hematoxylin-eosin (HE) staining were employed to detect *T. cruzi* in esophagi tissues from those patients. The blood parasitism was also identified through PCR, hemoculture and xenodiagnosis and correlated with tissue parasitism.

**MATERIALS AND METHODS**

**Patients.** Blood and tissue parasitism was evaluated in 52 chagasic patients with megaesophagus who had esophagomyotomy (Heller surgical procedure) with anti-reflux valve construction, at the Hospital Escola da Faculdade de Medicina do Triângulo Mineiro, Uberaba, in Minas Gerais, Brazil. The chagasic etiology of the megaesophagus was confirmed by three serologic tests: indirect immunofluorescence, indirect hemagglutination
and ELISA. Clinical classification of patients was done according to the results of esophogram, electrocardiogram, barium enema and/or number of days of constipation. The medical ethics committee of the Faculdade de Medicina do Triângulo Mineiro approved the project and all procedures were carried out with the informed consent of patients. Experiments were double-blind and patients were numbered chronologically in order of study entrance.

**Esophageal tissue collection.** During the megaesophagus surgery, a longitudinal strip of esophageal muscle (3-5 cm long by 1 cm wide) was excised from the lower portion of the esophagus near the gastric junction. Following washing with isotonic saline, the tissue was cut into fragments of approximately 1 cm² and frozen at -70°C. Upon thawing, the samples were divided into two groups, one of which was used fresh for DNA extraction and the other was fixed in absolute ethanol for histopathologic techniques. As a control, three esophageal samples were used from patients who underwent surgery for idiopathic megaesophagus who were negative by serology, xenodiagnosis and hemoculture for Chagas' disease.

**Extraction of tissue DNA.** For PCR, tissue samples were minced, subjected to alkaline lysis with 50 mM NaOH, heated in boiling water for 10 min, neutralized with 130 mM Tris-HCl (pH = 7.0) and used directly in PCR reactions after 10-fold dilution in double-distilled water.

**Histopathology and immunohistochemistry.** After fixing in absolute ethanol, tissue fragments were routinely processed for paraffin embedding. From those, two subsequentially cuts, slides were made, one of which was stained with hematoxylin-eosin to evaluate the inflammatory process and the other was used for the immunohistochemical PAP anti-*T. cruzi* parasite and/or antigen detection tests. The slides used for PAP were boiled in a microwave oven at maximum potential (700W) for 18 minutes. Anti *T. cruzi* serum, obtained from rabbit was used as a primary antibody (diluted 1:1,000, 4°C for 22 hours) followed by swine anti
rabbit IgG (Dako-Corporation, Carpinteria, CA, USA, diluted 1:100, 25° for 60 minutes) as secondary antibody. The reaction localization was made by the use of peroxidase-anti-peroxidase complex (Dako corporation, Carpinteria, CA, USA, diluted 1:100 for 60 minutes) followed by 0.048% DAB (Sigma-Aldrich, St. Louis, MO, USA) as chromogen. Slides were counterstained with hematoxylin.

**Evaluation of parasitemia using hemoculture and xenodiagnosis.** To detect circulating parasites in the blood of patients, xenodiagnosis was performed simultaneous with the collection of blood for culture and PCR. In general, this was done three days before and/or three days after the esophageal tissue fragment was collected. Hemoculture was done according to the methods described in the literature.¹⁵,²⁶ Immediately after collection, the 30 ml the blood was centrifuged at 4°C to remove the plasma. The packed cells were washed by centrifugation at 4°C in liver infusion tryptose (LIT) medium. The concentrate erythrocytes were re-suspended in 30 ml of LIT and uniformly distributed in six test tubes. Cultures were maintained at 28°C and homogenized weekly. Microscopic examination was done on the 30th, 60th and 90th days post-culture in 10 μl aliquots of suspension.

Each xenodiagnosis was performed with 40 third-stage nymphs of *Triatoma infestans* that were placed on patients' forearms for 30 min of feeding. Insects were individually examined for *T. cruzi* in the feces by abdominal compression and the content was examined by light microscopy 30th and 60th days after feeding.

**Blood collection for DNA extraction and PCR.** Blood (10 ml) was collected from chagasic patients for PCR reactions. The samples were immediately mixed with an equal volume of 6 M guanidine hydrochloride and 0.2 M EDTA solution, stored at room temperature for one week then at 4°C until processing.²⁷ Prior to DNA extraction the samples were boiled for 15 min to shear k-DNA molecules.²⁸ DNA extraction was done in duplicate with 200 μl of guanidine-EDTA-blood treated with an equal volume of phenol-chloroform,
chloroform and precipitate with two volumes of absolute ethanol, 0.3 M sodium acetate and
40 μg glycogen (Boehringer-Mannheim Biochemicals, Germany) according to the method
reported earlier.22

**PCR.** The protocol used to detect *T. cruzi* in blood and tissue samples was the same as
previously described,22 it consisted of specific PCR amplification of the 330 bp fragment,
corresponding to the four variable regions of *T. cruzi* kinetoplast minicircle DNA, followed
by slot-blot hybridization. PCR was carried out in a final volume of 20 μl containing 2 μl of
blood or tissue DNA solution; 10 mM Tris-HCl (pH = 9.0); 75 mM KCl; 3.5 mM MgCl₂;
0.1% Triton X-100; 0.2 mM of dATP, dCTP, dGTP and dTTP (Sigma-Aldrich, St. Louis,
MO, USA); 20 pmol of the primers 121: 5'-AAATAATGTACGGG(T/G)GAGATGCATGA-
3' and 122: 5'-GGTTGATTGCGGTGATATATA-3' (Operon Technology Inc,
Alameda, CA, USA); and 1.0 unit of *Taq* DNA polymerase enzyme (Promega, Madison, WI,
USA). The reaction mixture was overlaid with 30 μl of mineral oil and subjected to 35 cycles
of amplification in a MJ Research, Inc. (Watertown, MA, USA) Programmable Thermal
Controller (PTC-150). The temperature profile was 95°C for 1 min of denaturation with a
longer initial time of 5 min at 95°C, 65°C for 1 min for annealing of primer and 72°C for 1
min for extension, with final incubation at 72°C for 10 min to extend the annealed primers.
The PCR products were visualized in 6% polyacrylamide gel eletrophoresis and silver
stained.

**Slot-blot hybridization.** This technique was performed to confirm the *T. cruzi*
specificity of the amplicons observed in the polyacrylamide gels. In the hybridization
reaction, the S-67 5'-TGGTTTTGGAGGGGCGTTCAAATTT-3' probe recognized to the
sequences in the conserved region of the *T. cruzi* minicircle,29 detecting 0.1 fg of DNA.22 The
PCR products were denatured using 0.1 M NaOH and were applied to Biodyne B nylon
membranes (Life Technologies, Gibco-BRL, Gaithersburg, MD, USA) using a slot-blot
apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). The membrane was prehybridized for 1 hr at 55°C and hybridized for 30 min with alkaline phosphatase-labeled oligonucleotide probe S-67 that was synthesized by Lifecodes Corporation (Stanford, CT, USA). Detection was done using chemiluminescence with Lumi-Phos-Plus substrate (Life Technologies, Gibco-BRL, Gaithersburg, MD, USA) and X-ray film exposure as previously described.22

**Statistical analysis.** The EPIINFO-6 program was used to conduct comparison tests of simple proportions, the chi-square trend test for comparing the degree of megaesophagus with the *T. cruzi* in tissue and blood, and with inflammatory processes. Results were considered significant at $p < 0.05$.

**RESULTS**

**Clinical and epidemiological characteristics.** The clinical classification of the patients revealed that 23.1% (12/52) presented the digestive form exclusively: 17.3% (9/52) had only megaesophagus and 5.8% (3/52) had megaesophagus with megacolon. The cardiac form was encountered in 76.9% (40/52) of patients, in whom 48.1% (25/52) was associated with megaesophagus and 28.8% (15/52) with megaesophagus and megacolon. (Table 1) The megaesophagus varied from grade II to IV with 30.8% (16/52) of patients presenting in grade III and 34.6% (18/52) in grades II and IV. The average age of subjects was 55.9 ± 11.7 years; 69% (36/52) were males and 31% (16/52) females.

**Tissue parasitism.** PCR to detect *T. cruzi* DNA was performed randomly (i.e. without previously determining inflammatory foci) in the esophageal tissue fragments collected from the 52 patients. The *T. cruzi* kDNA 330 bp product was detected in 69.2% (36/52) of esophageal samples (Table 2), of which 25% (9/52) were confirmed only after hybridization (Figure 1). The PAP test was performed on tissue fragments adjacent to those used for PCR analysis and revealed the presence of the parasite and/or its antigens in 34.6%
tissue samples (18/52) Comparing the results of both tests, we found positive correlations in 26.9% (14 cases); however PAP was positive in 4 cases that had negative PCR. Using both techniques together resulted in the detection of *T. cruzi* in 76.9% (40/52) of the tissue samples from patients with megaesophagus.

**Comparison of the inflammatory process with the presence of parasite identified by immunoperoxidase and PCR.** An inflammatory process was observed in 50% (26/52) of the megaesophagus tissues. In 80.8% (21/26) we found an association with the presence of *T. cruzi*, its antigens and/or genomic fragments detected by PAP and PCR. When inflammation-free tissues were evaluated, *T. cruzi* was detected in 19/26 (73.1%) of the samples. To evaluate a direct association between inflammatory foci and presence of *T. cruzi* and/or its antigens, HE and PAP tests were performed in adjacent tissue cuts, resulting in a positive association in 46.2% (12/26) of the cases.

When the different degrees of megaesophagus were compared to parasitism and inflammatory processes, we found that grade IV cases had more frequent manifestations of both (Table 2), and *T. cruzi* was detected in 94.4% (17/18) of the tissues in which moderate to intense inflammation was present (Table 1). However, the Chi-square trend test did not reveal an association between increasing grades of megaesophagus and positive tissue parasitism detected by PAP+PCR ($\chi^2 = 1.38, p = 0.239$) nor with inflammatory processes ($\chi^2 = 2.724, p = 0.098$). But, a grade IV megaesophagus group was more likely to have a positive PAP test compared to the other groups ($\chi^2 = 7.704, p = 0.005$).

**Evaluation of blood parasitism.** Parasitemia was elevated in patients with megaesophagus who underwent corrective surgery. The parasite was recovered from circulating blood in 73.1% (38/52) of patients by xenodiagnosis and/or hemoculture. *T. cruzi* was identified in the blood of 75.0% (30/40) of the cases that had positive tissue parasitism and in 66.7% (8/12) of those with negative tissue parasitism.
PCR of blood amplified the 330 bp fragment corresponding to *T. cruzi* k-DNA in 90.4% (47/52) of subjects, of which 83% (39/47) was detected before hybridization of the amplified products. It was not possible to show a direct relationship between positive tissue and positive blood parasitism, because of 40 patients who had tissue parasitism, 92.5% (37/40) had positive blood PCR and *T. cruzi* was also detected in the blood of 83.3% (10/12) of the subjects with negative tissue parasitism. The grade of megaesophagus did not correlate with the distribution of positive results for the parasitologic (xenodiagnosis and hemoculture) and the PCR tests performed on the blood samples.

The controls, who had negative serology and no signs of chagasic infection, did not show amplified DNA products in either tissues or blood.

DISCUSSION

The guiding principle of clinical microbiology for over 100 years have been Koch’s postulates. According to these guidelines, to demonstrate an etiology the suspected agent must be present in affected individuals. In this study of 52 patients with chagasic megaesophagus, the presence of *T. cruzi* was found in 51 of them (98%) by PCR or culture proof. These data provide definitive evidence of long term persistence of *T. cruzi* in esophageal tissue and blood from patients with chagasic megaesophagus, and point to a key role of the parasite in unleashing the pathological processes in host.

The parasite, its antigens or its genomic fragments were detected randomly in 76.9% of the megaesophagus tissues, and were associated with inflammatory processes in 80.8% of these. The patients also presented increased levels of parasitemia during the tissue collection period: *T. cruzi* was detected in 90.4% by PCR and in 73.1% by xenodiagnosis and/or hemoculture, which could be interpreted as reflecting of accentuated tissue or blood parasitism.
The use of more sensitive techniques and the focused search for the parasite in selected areas with inflammatory lesions revealed a relationship between persistence of *T. cruzi* and disease severity in certain organs, both for cardiopathy and for megaesophagus. This association was seen in esophageal tissue in a small study in which PCR identified *T. cruzi* in four patients with megaesophagus but not in three who had cardiopathy. In endomyocardial biopsies of chagasic patients\textsuperscript{12} and in hearts from autopsies of severe chagasic cardiopathy cases,\textsuperscript{30} the parasite was detected by PCR in 91.3% and 94.7%, respectively, of the tissue samples adjacent to the inflammatory foci. The PAP test also revealed parasite antigens associated with inflammatory foci in cardiopathy cases, however no correlation was established between the quantity and intensity of the inflammatory process in tissue; severe cases had small amounts of antigen.\textsuperscript{13}

Despite its lower sensitivity (34.6%) compared to PCR (69.2%), the PAP test detected parasite antigens in four tissue samples that were negative by PCR. This discrepancy regarding PCR sensitivity may be explained by the parasite's focal distribution in the chronic phase of infection, which leads to parasites being in only one specific tissue region.\textsuperscript{31} An exhaustive search of *T. cruzi* by PAP was performed in eight chagasic patients with megaesophagi and in eight without, the parasite was only found in 50% of those with megaesophagus.\textsuperscript{32}

Some patients appeared to present elevated tissue parasitism since 75% of the positive cases had amplified more than 10 fg of DNA, which was detected by PCR alone. With hybridization it was possible to identify cases of DNA concentrations of up to 0.1 fg, suggesting lower tissue parasitism. Although several patients had blood and tissue DNA samples with differences in the intensity of the amplified PCR product, there was no evidence that this could account for the number of parasites present. Positive results and variability of *T. cruzi* DNA concentrations in tissues may be related to the presence of the parasite genomic
fragments inside host cells and inflammatory infiltrate cells. But, the k-DNA amplified from infected tissues is probably from recently liberated or dead parasites since inoculation of *T. cruzi* k-DNA in tissue of normal mice disappears after 48 hours.\textsuperscript{33}

Inflammatory lesions have been reported in 57% of esophageal-gastric junction biopsies in cases of megaesophagus and can vary from case to case and within the same patient depending on the point where the sample section is made.\textsuperscript{34} This finding agrees with our results in that 50% of the megaesophagus cases presented inflammatory alterations.

A correlation between the frequency and intensity of the inflammatory process in tissues and the presence of *T. cruzi* could be observed especially in cases of advanced megaesophagus. However, the detection of *T. cruzi* not associated with inflammation may be explained by the presence of intact nests of parasites.\textsuperscript{34,36} Such parasite nests were indicative of tissue invasion so recent that the local inflammatory process has not yet developed, or then are related to some degree of host immunosuppression, which has been shown in severe gastrointestinal forms\textsuperscript{37} and suggested in cardiac\textsuperscript{38} forms of Chagas' disease. The cellular immune response is an important factor in the control of *T. cruzi* in all stages of the disease and an immune imbalance could result in an increase in tissue and blood parasitism\textsuperscript{39}, a fact that is consistent with observations in the present study among chagasic patients with megaesophagus.

The sensitivity of the parasitologic tests, xenodiagnosis and hemoculture techniques could be related to the genetic characteristics of the *T. cruzi* strains, which vary according geographic region.\textsuperscript{42} This same situation seems to occur with PCR performed in blood where positive results in different chagasic populations and techniques fluctuate between 45 and 100%\textsuperscript{.20,22,41,42} The simultaneous application of two parasitologic tests increases diagnostic sensitivity,\textsuperscript{43,44} but PCR is more sensitive in detecting circulating parasites. Our data indicate 74.5% agreement between positive PCR in blood and the presence of circulating parasites
detected by indirect parasitologic tests, which is similar to published data.\textsuperscript{20,21} However, it was not possible to establish a direct relationship between the presence of the parasite in analyzed tissues and the patients' parasitemia.

As has previously suggested for the cardiac form, the parasite is probably playing an important role in the pathogenesis of esophageal lesions, particularly because of its continuous presence throughout the chronic phase. \textit{T. cruzi}, whether or not associated with lesions, is perhaps most responsible for the development of histopathologic changes because of ongoing immune response activation. This can be demonstrated by the elevated percentage of T lymphocytes (CD4$^+$ and CD8$^+$) expressing class II molecules of MHC (HLA-DR) in the peripheral blood of patients with the gastrointestinal form of Chagas' disease.\textsuperscript{37}

**Acknowledgments:** We are grateful to Dr Lúcia Maria da Cunha Galvão for help in PCR-hybridization, Orlando Carlos Magno, Afonso da Costa Viana from the Departamento de Parasitologia, Universidade Federal de Minas Gerais and Aparecida Correa, Helena Moraes, Isabel Moraes from the Departamento de Ciências Biológicas, Faculdade de Medicina do Triângulo Mineiro (FMTM) for technical assistance.

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CA = Clinical association; Inflam = inflammatory process; + (discrete); ++ (moderate); +++ (intense); ME = Megacalculus; C = Cardiac form; MC = Megalocon; ND = Not done; Hemox = hemoculture; Xeno = xenodiagnosis; Pos = positive; Neg = negative
## TABLE 2

Comparison of the grade of megaesophagus with tissue and blood parasitism and inflammatory processes

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ME = megaesophagus; PCR = polymerase chain reaction; PAP = peroxidase-anti-peroxidase; Inflamm = inflammatory process; Hemo = hemoculture; Xeno = xenodiagnosis; $p < 0.05$ = statistical significance; 

$\chi^2$ = Chi square (comparison of simple proportions among grades of megaesophagus)
FIGURE 1. Representative 6% polyacrylamide gel (A) and slot blot hybridization (B) from the same DNA esophageal tissue samples from patients with chagasic or idiopathic megaesophagus analysed by polymerase chain reaction (PCR). Lanes 1-4 and 7-10: The 330 bp fragment corresponding to T. cruzi k-DNA amplified by PCR from chagasic megaesophagus tissue samples and respective slot blots with strong positive hybridization; Lane 5: PCR product from an idiopathic megaesophagus no amplified in polyacrylamide gel and negative hybridization; Lanes 6, 11, 12: PCR from chagasic esophageal samples in which little or no T. cruzi k-DNA products were amplified, and where slight positive hybridization was observed.
CHAGASIC MENINGOENCEPHALITIS IN AN AIDS PATIENT: DIAGNOSIS, FOLLOW-UP AND GENETIC CHARACTERIZATION OF *Trypanosoma cruzi*

**Short title:** *T. cruzi* PCR and genetic characterization in Chagasic meningoencephalitis

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This work was conducted in accordance to Ethical Committee of Triângulo Mineiro Faculty of Medicine
Abstract

Early diagnosis of the clinical reactivation of Chagas’ disease in HIV-*Trypanosoma cruzi* co-infected individuals using specific and sensitive techniques is fundamental for a good prognosis. The polymerase chain reaction (PCR) rapidly and efficiently demonstrated the presence and elimination of *T. cruzi* in the cerebrospinal fluid of a chagasic meningencephalitis patient, making this a potential technique for diagnosis and therapeutic control in cases of central nervous system invasion. Preferential parasitic localization during reactivation may be associated with the exacerbation of specific sub-populations during the immunosuppression process. Characterization of *T. cruzi*, performed directly and indirectly in the blood and cerebrospinal fluid, using the low stringency single specific primer polymerase chain reaction (LSSP-PCR) and PCR amplification of sequences from the 24Sα rRNA gene, demonstrated homogeneity of k-DNA and the presence of lineage I (*T. cruzi* I) in both parasite populations. The definition of *T. cruzi* genetic standards in neurotropism and the use of PCR for monitoring parasites in the cerebrospinal fluid constitute a molecular approach which may lead to the adoption of new prophylactic conducts for patients with this co-infection.

**Key words:** *Trypanosoma cruzi*; AIDS; PCR; LSSP-PCR; rRNA.
Introduction

During the chronic phase of Chagas' disease Trypanosoma cruzi survive in low levels and may be recuperated from the circulating blood by indirect parasitological methods, such as hemoculture and xenodiagnosis. Reactivation of the infection does not occur spontaneously and has been associated with immunosuppression in transplanted patients [1, 2], hematologic cancer patients [3, 4] and those with acquired immunodeficiency syndrome (AIDS) [5-9].

Reactivation of chagasic infection in patients co-infected with the human immunodeficiency virus (HIV) has been related since 1990 [5] in approximately 60 well-documented cases [10]. The factors involved in this association are not clear and may be related to selective depletion of the host immune response or to the intrinsic characteristics of the parasite. These situations have been demonstrated by the presence of the parasite during microscopic examination of the buffy coat or microhematocrit [11, 12] and by the invasion of the central nervous system or the heart in the majority of cases [7, 8, 11]. Other less common manifestations have also been reported in the gastric and esophageal apparatus [8], skin lesions [13] peritoneum [14] and cervix uteri [15].

T. cruzi invasion of the central nervous system during the chronic phase of chagasic infection may be associated with the presence of sub-populations with specific characteristics. Experimental studies correlating phylogenetic divergences, histopathological alterations and tissue tropism have associated the genotype 20 of T. cruzi with central nervous system invasion [16]. Thus, it is important to evaluate if this parasite presents defined genetic profiles which may be associated with neurotropism.

The periodical control of parasitemia in co-infected patients should improve the management of clinical conduct and, subsequently increase survival rates [8, 12]. Current tests for diagnosis of central nervous system invasion in Chagas' disease have a low sensitivity and in some situations do not allow the establishment of etiology. The use of rapid and specific techniques such as the polymerase chain reaction (PCR) which detects minimal quantities of the parasitic DNA [17, 18] may constitute a useful tool for early diagnosis of the disease and for accompaniment of treatment.

This investigation demonstrates the applicability of molecular techniques in the detection of T. cruzi in the blood and cerebrospinal fluid and in the genetic characterization of the parasitic population associated with central nervous system invasion in humans.
Methods

Case Report

A 63-year-old male patient from Minas Gerais, Brazil was hospitalized with a history of fever lasting several days, general malaise, disseminated lesions on the body compatible with psoriasis and HIV-positive serology. Three days after hospitalization the patient presented monoparesis on the left side which rapidly developed into hemiparesis. A computed tomographic scan of the brain demonstrated a cerebral lesion on the right side with a remarkable mass effect, collapsing of the ipsilateral ventricle without capture of the contrast fluid. The diagnostic hypothesis was of cerebral toxoplasmosis and a therapeutic conduct of sulfadiazine and pyrimetamine was initiated. Despite treatment the clinical situation evolved to mental confusion, intense psychomotor agitation, nuchal rigidity and paralytic midriasis on the right side. At that time the T lymphocytes count was 67 CD4⁺ cells/mm³ and 313 CD8⁺ cells/mm³ and the electrocardiograph showed left atrial overload, left ventricular overload and diffuse alteration of ventricular repolarization. The diagnosis of chagasic infection and reactivation was made 6 days after patient admission by the use of the indirect immunofluorescence reaction in the serum and by demonstration of parasites in the cerebrospinal fluid and blood. A therapy with benznidazole (7mg/kg/day for 60 days) was immediately started but the patient fell into a profound coma 3 days later. Control of treatment was performed by parasitological evaluation in the cerebrospinal fluid and cranial tomography at 7 and 22 days. Sixty eight days later the patient was discharged from hospital still in a comatose state and demonstrating just primitive reflexes such as suction and holding. At the time of hospitalization the patient signed a consent form authorizing all necessary procedures during his hospitalization and their use for teaching and research.

Evaluation of parasitism in blood and cerebrospinal fluid: Cerebrospinal fluid and blood collection from the patient was performed simultaneously and was carried out initially to confirm the diagnostic hypothesis (T0) and later to accompany the development of the patient at 7 (T1) and 22 days (T2) after the initiation of treatment. These samples were submitted to direct microscopic examination, microhematocrit, culture in LIT medium and PCR for the detection of the parasite and or its DNA. Hemoculture was performed using 10 ml of blood in liver infusion tryptose (LIT) medium according to the methodology previously described [19]. Cerebrospinal fluid culture was performed by the addition of 1ml aspirated fluid directly to 5ml of LIT medium. Cultures were maintained at 28°C and homogenized weekly. Microscopic examination was performed on the 30th, 60th and 90th days after culture.
Blood and cerebrospinal fluid collection for DNA extraction and PCR: Total blood (10 ml) and cerebrospinal fluid (2ml) were collected for PCR evaluation. The samples were immediately mixed with an equal volume of 6 M guanidine hydrochloride and 0.2 M EDTA solution [17]. Negative samples by direct or microhematocrit examination were boiled before extraction of the DNA for 15 min to break the minicircles molecules [20], for all other samples the DNA was extracted directly without previous boiling. DNA extraction was carried out in duplicate with 200 µl of guanidine-EDTA-blood according to a previously described method [18].

Extraction of isolated T. cruzi DNA from blood or cerebrospinal fluid culture: The blood or cerebrospinal fluid culture samples were diluted 1:1 in guanidine-EDTA and DNA extraction was performed, without previous boiling, according to the previously described method.

PCR amplification of T. cruzi kinetoplast minicircle DNA (k-DNA): The protocol used to detect T. cruzi in blood, cerebrospinal fluid and culture samples was essentially the same as previously described [18] and consisted of the specific PCR amplification of the 330 bp fragment corresponding to the four variable regions of T. cruzi k-DNA using 121: 5'-AAATAATGTACGGGT(T/G)GAGATGCATGA-3' and 122: 5'-GGTTGCATGGGGTTGGTAAATATA-3' (Operon Technology Inc, Alameda, CA, USA) primers. PCR products were visualized by 6% polyacrylamide gel electrophoresis and silver stained. For the cerebrospinal fluid samples, PCR was performed using successively diluted DNA samples until no amplified products could be detected in the polyacrylamide gels and hybridization. For the samples in which no PCR amplification was detected, the presence of amplification inhibitors was verified by the addition of 0.1 ng of parasite DNA (obtained by culture in LIT medium) to samples at the time of PCR.

Slot-blot hybridization: This technique was performed using PCR-amplified DNA (k-DNA) samples of blood and cerebrospinal fluid to confirm T. cruzi specificity for the amplicons observed in the polyacrylamide gels. In the hybridization reaction, the S-67 5'-TGGTTTTGGGAGGGGCGTTCAAATTT-3' probe attached to the sequences in the conserved region of the T. cruzi minicircle [21], detecting as little as 0.1 fg DNA [18]. The PCR products were denatured and were applied to a nylon membranes (Biodyne B Life Technologies, Gibco-BRL, Gaithersburg, MD, USA) using a slot-blot apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). The membrane was hybridized for 30 min with the alkaline phosphatase-labeled oligonucleotide probe, S-67 (synthesized by Lifecodes Corporation, Stanford, CT, USA). Detection was carried out by chemiluminescence using a Lumi-Phos-Plus substrate (Life Technologies, Gibco-BRL, Gaithersburg, MD, USA) and X-ray film exposure as previously described [18].
Low stringency single specific primer polymerase chain reaction (LSSP-PCR): The kinetoplast genetic characterization of parasites was performed directly from the samples and using cultures samples obtained from blood or cerebrospinal fluid collected before the initiation of treatment (T0) and one week later (T1). This technique was performed using a two-step procedure. The first consisted of PCR amplification of the k-DNA minicircle 330-bp fragment, as described previously. The PCR amplified products were then submitted to electrophoresis in 1.5% agarose gel (1.0% agarose, 0.5% low melting point agarose) stained by ethidium bromide. The bands corresponding to the 330-bp fragment were visualized by long-wave ultraviolet and excised from the gel. These were diluted 1:10 in double-distilled water and submitting to a second step of low-stringency amplification (LSSP-PCR) [22] using a single 121 modified primer (21bp) also known as S35G: 5′-AAATAATGTACGGGGGAGATG-3′ (Operon Technology Inc, Alameda, CA, USA). The LSSP-PCR products were visualized by 7.5% polyacrylamide gel electrophoresis and silver stained. The control of this reaction was done using the CI strain of T. cruzi.

PCR amplification of 24Sα rRNA sequences: PCR amplification of a divergent domain of the 24Sα RNA gene was achieved with D71: 5′-AAGGTGCCTGACGTGGTGG-3′ and D72: 5′-TTTTCAATGCGCCAGCTGTGG-3′ (Operon Technology Inc, Alameda, CA, USA) in the samples of cultures from blood and cerebrospinal fluid collected at T0, following protocols described previously [23]. PCR products were visualized by 6.0% polyacrylamide gel electrophoresis and silver stained. The amplification of a fragment of 125-bp corresponded to the lineage 1 and a fragment of 110-bp corresponded to lineage 2, currently known as T. cruzi II and T. cruzi I, respectively [24].

Results

Parasitological evaluation: Examination of fresh samples collected at T0 demonstrated 1×10^3 parasites/ml in the blood and uncountable trypanosomes T. cruzi forms in the cerebrospinal fluid, indicating strong invasion of the central nervous system. Treatment with benznidazole induced a dramatic reduction in the parasitic level in both the blood and the cerebrospinal fluid; at T1 T. cruzi was detected by hematocrit and at T2 the parasite was not detected by any of the direct methods utilized. The blood and cerebrospinal fluid cultures were parasite positive at T0 and T1 and negative at T2.

PCR and PCR-hybridization in the cerebrospinal fluid and the blood samples: The k-DNA of T. cruzi was strongly amplified in the cerebrospinal fluid at T0 and T1. Despite the reduction in parasite levels after treatment, the quantity of DNA in the cerebrospinal fluid remained high at T1 with levels similar to those found
at T0. These levels were measured by PCR-hybridization which demonstrated products corresponding to the amplification of T. cruzi k-DNA up to dilutions of $10^{-7}$ and $10^{-8}$, respectively for T0 and T1. These products were detected using polyacrylamide gel in dilutions of up to $10^{-6}$ and $10^{-7}$, respectively for T0 and T1. (Figure 1). T. cruzi clearance was observed at T2 and its presence was only confirmed by weakly positive hybridization at most concentrate dilutions in two of the four extractions of DNA from previously boiled samples (Figures 1 and 2). No inhibitors were detected in these samples and the negative results were seen to indicate the absence of T. cruzi DNA.

The DNA samples obtained from blood were diluted up to $10^{-4}$ and PCR strongly amplified T. cruzi k-DNA in T1 and T2 as well as in T0.

**LSSP-PCR reaction:** The genetic characterization of T. cruzi using a polymorphic study of the hypervariable region of the T. cruzi minicircle utilizing LSSP-PCR revealed identical genetic signature profiles in the blood and in the cerebrospinal fluid before treatment (T0) and 1 week later (T1), suggesting the presence of the same parasitic population. The genetic homogeneity of the parasite was also observed in the band profiles generated by the parasites present in the culture isolates (Figure 3). The T. cruzi CL strain used as a control for LSSP-PCR presented its own band profile which differed to that of the study (Figure 3). **PCR amplification of the 24Sα rRNA gene sequences:** The T. cruzi population isolated from this patient, in both the blood and the cerebrospinal fluid, corresponded to the parasitic lineage I (T. cruzi II) associated with the domestic transmission cycle, with products amplified in the 125-bp region.

**Discussion**

Although regional endemic diseases are not classified among the infections associated with HIV/AIDS in the Centers for Disease Control and Prevention (CDC) of Atlanta, the opportunistic character of Chagas' disease [8, 10] is illustrated by the increased number of parasites present in both the blood and the cerebrospinal fluid and the low number of T CD4⁺ lymphocytes (67 cells/mm³). The number of individuals co-infected with HIV and T. cruzi is unknown, although data presented at scientific events and confirmed by necropsy indicate that these individuals make up a greater proportion of those in which chagasic infection is reactivated, only 6 to 16.7 % [10, 12]. Highly active anti-retroviral therapy (HAART) and the use of azolic derivatives in anti-fungal therapy probably contributes to the low rate of reactivation in these patients.

Parasitemia seems to perform an important role in the reactivation of Chagas' disease and may precede the clinical manifestations or be detected later on [11, 13, 25] being greater in patients with the clinical form of
chagasic meningoencephalitis than in patients with the myocardial form [26]. The speed of T. cruzi detection at the beginning of specific treatment may reduce its multiplication and impede the dissemination of the infection by the central nervous system and the heart [25]. In the cases of central nervous system invasion, clinical-tomographic similarities with neurotoxoplasmosis may make diagnosis more difficult [8, 27, 28], delaying the initiation of an adequate therapy.

The drugs available for treatment of chagasic meningoencephalitis, nifurtimox and benznidazole and their association with imidazolic derivatives are not always effective and the outcome is variable [7, 8, 12, 27, 29, 30]. Therapeutic control is based on the reduction and disappearance of the lesions (visualized by tomography), on the elimination of the parasite in the cerebrospinal fluid and the subsequent clinical improvement of the patient. The diagnostic techniques and the control of parasitism, such as xenodiagnosis and LIT medium culture, although specific are slow and have low sensitivities. In addition, biopsy is an invasive procedure. These techniques may give false-negative results in cases of low level parasitism making evaluation of the patient’s clinical state inconclusive and slow.

The data herein presented show that in the case of central nervous system invasion, PCR demonstrated an excellent capacity for detecting T. cruzi and controlling treatment since it clearly demonstrated the presence and elimination of the parasite in the cerebrospinal fluid. A positive PCR in the cerebrospinal fluid suggests the presence of an intracellular T. cruzi cycle in the cerebrum and/or its recent destruction. The increased concentration of DNA in the cerebrospinal fluid associated with the decrease of parasitism observed 1 week after the initiation of treatment (T1) seems to be a reflection of intense destruction of the parasites by the action of benznidazole. The use of PCR in the monitoring of T. cruzi during treatment may be a rapid and safe indicator of the susceptibility of the parasite to the action of the drugs, allowing early changes in the therapeutic conduct in cases of resistance. In this case, although the treatment with benznidazole was initiated late on and its tissue action was not evaluated, its capacity to suppress T. cruzi in the cerebrospinal fluid and reduce parasitemia was significant as evidenced by the parasitological examinations and PCR.

The time of T. cruzi DNA persistence in the vertebrate host is still undetermined, some experimental evidence exists to suggest that it is eliminated from the blood circulation within 48 hours [31]. In this study, the weak amplification of residual DNA observed at T2 when compared with T1 suggests that clearance in humans may take at least 15 days.

The diversity of clinical manifestations of human Chagas' disease has been attributed to the characteristics of the host immune response and to the genetic heterogeneity of the parasite which may be
formed by a multiclonal population with various biological profiles [32, 33, 34]. The preferential location of T. cruzi in the central nervous system is still unclear and may be associated with the presence of sub-populations with neurotropical characteristics which may be repressed by the immunological response during the acute phase of infection and later reactivated during the immunosuppression of the host. Experimental evidence demonstrated that after immunosuppression in mice in the chronic phase of chagasic infection, new sub-populations of T. cruzi with different genetic properties may emerge with subsequent development of meningoencephalitis [35].

Other published data, however, indicate that immunosuppression resulting from HIV infection does not lead to the establishment of new T. cruzi genotypes, since T. cruzi stocks isolated from these patients were closely related to clonal genotypes previously identified (30 or 32) in 89 % and 94 % of the stocks isolated from HIV+ and HIV− patients, respectively [26].

In this investigation, the genetic characterization of T. cruzi k-DNA performed directly in the cerebrospinal fluid and blood of the patients using LSSP-PCR permitted a true analysis of the parasitic population involved in the pathological process. The fact that the genetic signature profiles found in the population in the central nervous system and the blood were identical suggests that, in this case at least, immunosuppression did not induce exacerbation of different sub-populations capable of selective neurotropism. The homogeneity of the band profiles generated by LSSP-PCR before and one week after treatment also indicated that, at least during this period, benznidazole did not induce selection of parasitic sub-populations. The same was seen in the parasites of the culture medium isolates. The possibility that the sequence studied corresponds to a monoclonal population, though, should not be excluded and would explain the result obtained.

The characterization of the T. cruzi sequence of the 24Sα rRNA gene allowed T. cruzi to be divided into 2 groups or lineages corresponding to the domestic cycle, lineage 1 (T. cruzi II) and to the sylvatic cycle, lineage 2 (T. cruzi I), the biological significance of which is under discussion. Experimentally, just the T. cruzi genotype 20 appears to be associated with central nervous system invasion [16], this genotype belongs to lineage 2 [36] and its multiplication is easily controlled by the host immune system [37]. The T. cruzi strain evaluated in this study demonstrated it to be lineage 1 (T. cruzi II), predominant in human infections of both the blood and the cerebrospinal fluid and also frequent in the endemic area from which the patient originated [38]. It has been suggested that the strains of T. cruzi associated with invasion of the central nervous system may be genetically similar to the CL strain [39]. However, whilst the strain studied and the CL both belong to lineage 1, the patterns of their genetic signatures determined by LSSP-PCR were different, indicating that their minicircles, i.e. their k-
DNA, possess distinct sequences. These data reinforce the necessity for further studies characterizing the sub-populations of *T. cruzi* for the definition of the genetic profiles associated with the differentiated tropisms [34], such as neurotropism.

Adequate monitoring of *T. cruzi* in patients carrying sequences with the genetic potential to invade the central nervous system may possibly evict future cases of chagasic disease reactivation in patients co-infected with HIV. PCR is a sensitive, specific and fast technique for the detection of *T. cruzi* in the cerebrospinal fluid and may be an effective tool for the early diagnosis and accompaniment of treatment of these patients and should be further investigated.
References


Legends

Figure 1: Polyacrylamide gel (6%) showing the PCR amplification of *T. cruzi* in the cerebrospinal fluid over time. The presence and subsequent elimination of the parasite during treatment can be observed. DNA samples were collected before (T0) and during treatment at 7 (T1) and 22 days (T2). Lines 1-7: DNA obtained at T0 and diluted from $10^{-2}$ to $10^{-8}$, demonstrated amplification of a 330-bp band up to the $10^6$ dilution corresponding to the DNA of *T. cruzi*; Lines 8-15: DNA at T1 diluted $10^{-1}$ to $10^8$, amplified products visualized up to the $10^7$ dilution; Lines 16-19: DNA at T2, pure and diluted $10^{-1}$ to $10^{-3}$ with weak and doubtful *T. cruzi* kDNA amplification; MW: molecular weight.

Figure 2: Slot-blot demonstrating hybridization of the PCR amplified products in the cerebrospinal fluid at 7 (T1) and 22 days (T2) of treatment. Slots 1-2 positive are positive controls and 21-22 are negative controls; Slots 3-11: PCR of DNA of cerebrospinal fluid at T1, pure and diluted from $10^{-1}$ to $10^8$, demonstrating the presence of *T. cruzi* DNA up to the $10^8$ dilution. Slots 12-20: PCR of DNA of cerebrospinal fluid at T2, pure and diluted from $10^{-1}$ to $10^8$, weakly showing the presence of *T. cruzi* DNA in the most concentrated samples.

Figure 3: Comparison of the genetic profiles (LSSP-PCR) of the *T. cruzi* populations present in the blood and cerebrospinal fluid before treatment (T0, lines 1-2), at 7 days of treatment (T1, lines 3-4), hemoculture performed at T0 (line 5), CL strain culture (line 6); MW: molecular weight. Populations were visualized in a 7.5% polyacrylamide gel and silver stained.
FIGURE 1.
XIV Reunião Anual de Pesquisa Aplicada em Doenças de Chagas

SESSÃO INAUGURAL
CONFERÊNCIA: VACINAÇÃO EM LEISHMANIOSES: ANTÍGENO RECOMBINANTE QUE CONFERE PROTEÇÃO TOTAL EM MODELO MURINO

Conferencista: Antônio Campos Neto (EEUU)
Presidente: Valdemar Hial (MG)

7/11/98 - Sábado
8:00 às 9:30 - Auditório A

MESA REDONDA: A DIVERSIDADE DO PARASITA E HOSPEDEIRO NA DOença DE CHAGAS

Presidente: José Rodrigues Coura (RJ)
Secretário: Mario León Silva-Vergara (MG)
- Izabel K. Miranda Santos (DF)
- Eliane Lages Silva (MG)
- Estrutura clonal das cepas do T. cruzi: importância de clones principais - Sonia Gumez Andrade (BA)
- Antônio Teixeira (DF)

8:00 às 9:30 - Auditório B

MESA REDONDA: IMUNOPATOLOGIA DAS LEISHMANIOSES

Presidente: Washington Luiz Tafuri (MG)
Secretário: Dalmo Correia Filho (MG)
- Hematogenic dissemination, parasite persistence and immune evasion in human leishmaniasis. Claude Pirmez (RJ)
- Sérgio Coutinho (RJ)
- Edgar Marcelino Carvalho (BA)
- Análise comparativa do diagnóstico da LTA por PCR, usando como fonte de DNA do parasito biópsias e "imprint" das biópsias em papel de filtro e de nitrocelulose - Álvaro José Romanha (MG)

9:30 às 9:45 - Café

XI Reunião Anual de Pesquisa e Aplicada em Doenças de Chagas e III Reunião de Pesquisa Aplicada em Leishmanios - 5 a 7/11/1999 - Uberaba, MG

Kenneth J. Gollub (Instituto de Ciências Biológicas, UFMG, MG)
Manoel Otávio Rocha (Universidade Federal de Minas Gerais)
Marcelo Lorenzo (Centro de Pesquisa Gonçalo Moniz, RJ)
Marcelo Simão Ferreira (Faculdade de Medicina Uberlândia, MG)
Marco Tulio A. G. Zapata (Inst. Patologia. Trop. e Saúde Pública, GO)
Marília Terezinha Bahia (Universidade Federal de Ouro Preto, MG)
Mário León Silva (Faculdade de Medicina do Triângulo Mineiro, MG)
Marta Teixeira (Instituto Ciências Biomédicas, USP)
Paulo César Cotrim (Faculdade de Medicina USP, SP)
Pedro Luiz Pinto Silva (Instituto Adolfo Lutz, SP)
Ricardo Ribeiro Santos (Centro de Pesquisas Gonçalo Moniz, RJ)
Sérgio C. Mendonça (Instituto Oswaldo Cruz, Fiocruz, RJ)
Sérgio Salles Xavier (Instituto Oswaldo Cruz, Fiocruz, RJ)
Sheila Jorge Adad (Faculdade de Medicina do Triângulo Mineiro, MG)
Tânia C. Araujo-Jorge (Instituto Oswaldo Cruz, Fiocruz, RJ)
Walter Goleman (Universidade Federal do Rio de Janeiro, RJ)

13:30 às 14:45h - DISCUSSÃO DE POSTERS

14:45 às 15:00h - Café

15:00 às 17:30h

MESAS REDONDAS

Anfiteatro A: IV- Patogênese da doença de Chagas: o papel das cepas e do hospedeiro

Presidente: Jofre Marcondes de Resende (Faculdade de Medicina da Universidade Federal de Góias)
Moderador: José Roberto Mineo (Faculdade de Medicina Uberlândia, MG)

1. Trypanosoma cruzi: TC₁ e TC₂: Bianca Zingales (Instituto Química, USP, SP)
2. Biotipos de T. cruzi - resistência a drogas e controle pós-terapêutico na doença experimental - Sónia Andrade (Instituto Pesquisas Gonçalo Moniz, Fiocruz, BA)
3. Citocinas nas formas cardíaca e indeterminada - Juliana de Assis (Instituto Pesquisas René Rachou, MG)
4. Aspectos imunológicos na forma digestiva - Rodrigo Correa (Instituto Pesquisas René-Rachou, Fiocruz, MG)
5. Variabilidade genética do Trypanosoma cruzi e a patogênese da Doença de Chagas - Sérgio Danillo J. Pena (Universidade Federal de Minas Gerais, MG)
6. Diversidade de Trypanosoma cruzi no megaestágio chagásico - Eliane Lages Silva (Faculdade de Medicina do Triângulo Mineiro, Uberaba, MG)

Debatedores:
   Andrea Macedo (Instituto de Ciências Biológicas, UFMG, MG)
   Carlos Corbett (Faculdade de Medicina da USP, SP)

Rev. Soc. Bras. Med. Trop. 32(Supl. II) 1999
MB-37 – CHAGAS DISEASE REACTIVATION IN AIDS PATIENTS: TREATMENT CONTROL USING PCR AND GENETIC CHARACTERIZATION OF SUB-POPULATIONS OF T. CRUZI IN BLOOD AND CEREBRAL FLUID

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The opportunistic character of Trypanosoma cruzi has been reported mainly in cases of co-infection with the human immunodeficiency virus. In such cases, chagas infection reactivates, invades the central nervous system (CNS) and has high lethality. The factors involved in this process are unknown but appear to depend on host immune response and characteristics common to certain parasite populations. This study follows one case of Chagas disease reactivation in an AIDS patient. T. cruzi genetic characteristics as well as blood and cerebral fluid parasitemia before and during benznidazole treatment were analyzed. The patient is a 63-year-old male with the following profile: 1,000 leukocytes/mm³, CD4+ 67/mm³ and CD8+ 313/mm³. He was hospitalized in HE-FMTM with meningoencephalitis that did not respond to the initial treatment for toxoplasmosis. Later, T. cruzi was detected in blood and cerebral fluid and the patient was treated with benznidazole (Tmg/Kg) for 60 days. Blood and cerebral fluid samples were collected before treatment (T0), and at 7 (T1) and 22 (T2) days post-treatment. Direct, microhemocytocrit, LIF medium culture, PCR and LSSP-PCR examinations were performed on the samples. Direct and microhemocytocrit evaluations indicated higher parasitemia in the cerebral fluid than the blood at T0, dropping sharply in T1 and being negative in T2. Cultures of cerebral fluid and blood were positive in T0 and T1 but were negative in T2. T. cruzi DNA was amplified by PCR in dilution higher than 1:200 for cerebral fluid and blood in both T0 and T1. In T2, we verified that blood continued to be strongly positive in PCR analyses while DNA amplification in cerebral fluid was weak and only occurred in undiluted samples (negative in 1:10; 1:100 and 1:200 dilutions), which suggests the disappearance of circulating parasites in this fluid. Using the LSSP-PCR technique, we genetically characterized sub-populations of parasites present in the cerebral fluid and blood and observed genetic signatures with profiles that indicated a single population. PCR is a helpful tool for early detection of the parasite in cerebral fluid and for evaluating treatment efficacy in patients with reactivated infections of the CNS. It is necessary to define genetic patterns of T. cruzi populations related to CNS involvement in order to determine preventive measures for immunosuppressed patients.

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MB-38 – PCR-BASED DIAGNOSIS FOR CHAGAS' DISEASE IN TREATED OR NON-TREATED PATIENTS LIVING IN ENDEMIC AREA

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In recent years, various investigators reported the use of the polymerase chain reaction (PCR) in the diagnosis of chronic chagasic infection, and a variety of T. cruzi-specific amplification target sequences have been described. Most of the published studies use kDNA minicircle sequences as target molecules, due to their abundance in the parasite genome. Each parasite contains a network of approximately 20,000 concatenated minicircles, each of which possesses four copies of target sequences. When compared with xenodiagnosis or hemoculture the PCR technique has shown a consistent increase in sensitivity. However, low sensitivity of PCR in comparing serologic tests has also been reported. Because of these disparities, the true and potential usefulness of PCR for chronic Chagas disease diagnosis further investigation.

In this study we analysed 87 specimens from individual living in endemic area for Chagas disease, Virgem da Lapa, MG. Some patients were submitted to a specific chemotherapy. Total DNA was isolated by phenol/chloroform extraction followed by ethanol precipitation. The primers 121/122 were designed to amplify T. cruzi kentoplast DNA minicircle sequence (kDNA). The amplified products were analysed by agarose gel electrophoresis, ethidium bromide staining and by enzyme-linked immunosorbent assay (ELISA).

In substitution of the hot start method, we utilized the Platinum Taq DNA polymerase (GIBCO). Platinum Taq DNA polymerase is derived from recombinant Taq DNA polymerase by binding of a thermolabile inhibitor containing monoclonal antibodies to Taq DNA polymerase to provide automatic hot start.

The PCR technique was compared with some serological methods, indirect immunofluorescence (IFI), ELISA, western blotting (WB) and INNOLIA.

The results of serological methods showed 12 negative samples and 75 positive samples. All the negative samples serologically, were negative in PCR. The PCR technique detected 50 of them 75 positive samples serologically only.

XXVI Annual meeting on Basic Research in Chagas' Disease
9-11/11/1989
Caliambre, MG.
IM-21 – EXPERIMENTAL T. CRUZI INFECTION: II. ROLE OF TNF-α, INF-γ AND IL-10 IN THE PROTECTION OF MICE IMMUNIZED WITH HEMOCYTES FROM UNINFECTED T. INFESTANS

Levy, A.M.A.1, Lourenço, A.M.1, Pereira-Chioccola, V.L.1, Fragata-Filho, A.A.1, Nunes, E.V.2 & Hoshino-Shimizu, S.2 1. Instituto Dante Pazzanese de Cardiologia, Av. Dr. Dante Pazzanese, 500, CEP 04012-180 São Paulo, SP, Brazil; 2. Instituto Adolfo Lutz

Hemocytes (HC) from uninfected tritomines were shown to interact with chagasic sera. Moreover in immunized mice with HC from uninfected T. infestans followed by a challenge of trypomastigotes of “Y” strain, a partial protection was observed with low parasitemia and mortality. However, sera from these mice before the challenge failed to recognize both trypomastigotes as well as epimastigotes. Since the humoral immune response was not detected, the involvement of cellular response was here studied. Female A/Sn mice were immunized using alumen’s adjuvant with HC from uninfected 5th instar T. infestans nymphs, three times with 10-15 day intervals. The group I of mice received successively 2, 3 and 4 x 10³ HC/mL. The group II received 188-200μg protein of hemolymph (HL) free of HC and the group III (control) received only alumen’s adjuvant (0.5mg/mL). All groups of mice were challenged with 5 x 10⁶ bloodstream trypomastigotes 15 days after immunization. Sera were collected before T. cruzi infection, before the peak of parasitemia, shortly after the peak and in the chronic phase. TNF-α, INF-γ and IL-10 were assayed by ELISA (Biosource). HL-mice presented higher level of INF-γ before the challenge than the other groups, followed by a significant decrease and kept low values until the end of the experiment. HC-mice, however, showed higher value than the control group before the challenge. Then, after a slight fall before the peak (like the control group) the level increased at the peak and in the chronic phase, in contrast to the control group. In respect to the TNF-α, HL-mice showed a similar profile to the control group, whereas in the HC-mice, there was a remarkable increase towards the peak, decreasing slightly thereafter. The IL-10 in turn was high in the control group, low in HL-mice and significant lower in HC mice. The data demonstrate that the partial immunoprotection is provided by HC cells rather than HL, through the mechanisms of cellular immunity.

IM-22 – ASSOCIATION OF MEGAESOPHAGUS WITH BLOOD AND TISSUE PARASITEMIA IN CHRONIC CHAGASIC PATIENTS USING PCR, HEMOCULTURE AND XENOCLONALYSIS

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The mechanisms involved in the development of lesions in Chagas disease are not well understood. However, the persistence and density of parasites in tissues appear to be associated with the evolution of different clinical forms. Here we evaluate blood and tissue parasitemia in 50 chagasic patients with megaesophagus who underwent therapeutic surgery. Esophageal fragments from three individuals with idiopathic megaesophagus were used as controls. The following tests were performed: hemoculture, xenodiagnosis and PCR using S35 and S36 primers to amplify the 330-bp region of Trypanosoma cruzi kDNA. In blood, parasite DNA was detected in 76% of patients using xenodiagnosis or hemoculture and in 81.6% using PCR. In tissue fragments, the presence of parasite DNA was observed in 50% of samples and DNA extraction was random, that is, not associated with an inflammatory focus. Of the patients that had positive PCR results in tissues, 84% had positive parasitologic and PCR results in blood. Among patients that had negative PCR results in tissues, 79.16% had positive PCR results in blood while the parasite was detected in 68% of them using xenodiagnosis or hemoculture. T. cruzi is easily detected by PCR in blood of patients with both positive and negative PCR in tissues, with no correlation observed between the presence of the parasite in tissue and in blood. The high parasitemia observed in subjects suggests some type of immunosuppression related to this clinical form or could reflect an intrinsic characteristic of T. cruzi populations associated with megaesophagus. The presence of T. cruzi DNA in esophageal tissue illustrates the importance of the parasite in the evolution of chagasic megaesophagus.

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