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kDNA gene signatures of *Trypanosoma cruzi* in blood and oesophageal mucosa from chronic chagasic patients

Fernanda da Silva Manoel-Caetano[a], Cláudia Márcia Aparecida Carareto[a], Aldenis Albaneze Borim[b], Kenji Miyazaki[c], Ana Elizabete Silva[a,*]

[a] *Department of Biology, UNESP - São Paulo State University, São José do Rio Preto, SP, Brazil*
[b] *Department of Surgery, FAMERP - School of Medicine, São José do Rio Preto, SP, Brazil*
[c] *Service of Endoscopy, Hospital de Base, São José do Rio Preto, SP, Brazil*

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Summary *Trypanosoma cruzi* presents a high degree of intraspecific variability, with possible implications for the pathogenesis of Chagas disease. The aim of this study was to evaluate *T. cruzi* kDNA minicircle gene signatures using the low-stringency single-specific-primer PCR technique in both peripheral blood and oesophageal mucosa from chronic chagasic patients, with or without megaesophagus, alone or in combination with cardiopathy and megacolon. It was not possible to identify a uniform pattern of shared bands between blood and oesophageal mucosa samples from individuals with the same clinical form or mixed forms, suggesting multiple *T. cruzi* infections with differential tissue tropism. Thus, the results indicate that there is an intense intraspecific variability in the hypervariable regions of *T. cruzi* kDNA, which has so far made it impossible to correlate the genetic profile of this structure with the clinical manifestations of Chagas disease.

1. Introduction

Trypanosoma cruzi is the etiological agent of Chagas disease, a frequent anthropozoonosis afflicting almost 18 million people throughout Central and South America (Prata, 2001). Chagas disease is characterized by a wide spectrum of clinical outcomes, ranging from absence of symptoms to severe disease with cardiac involvement and/or digestive tract damage, such as megaesophagus and megacolon (Gomes et al., 2003). Geographic variation in the prevalence of clinical forms and morbidity of Chagas disease has been reported (Dias, 1992). In Brazil, the asymptomatic or indeterminate form is the most common (60—70%), followed by the cardiac (20—30%) and digestive (8—10%) forms. However, in central Brazil and Chile the digestive

* Corresponding author. Present address: Universidade Estadual Paulista, Departamento de Biologia, Rua Cristóvão Colombo 2265, CEP 15054-000, São José do Rio Preto, SP, Brazil.
Tel.: +55 17 3221 2384; fax: +55 17 3221 2390.
E-mail address: anabete@ibilce.unesp.br (A.E. Silva).

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form of the disease predominates, while it is virtually nonexistent in Venezuela and Central America (Dias, 1992).

The reasons for this geographic heterogeneity and for the different clinical forms developed by different patients are still unexplained. It is generally believed that they are primarily determined by a genetic variation of *T. cruzi*, but a role for environmental, nutritional and immunological aspects of the host is possible in establishing the clinical type of the disease (Macedo et al., 2004). *Trypanosoma cruzi* populations present an intense intraspecific variability as demonstrated by different biochemical and molecular techniques (Brisse et al., 2000; Macedo et al., 2001), but this polymorphism has not yet been correlated with the pathogenesis of Chagas disease (Lages-Silva et al., 2006).

The low-stringency single-specific-primer PCR (LSSP-PCR) technique has allowed the genetic characterization of *T. cruzi* by a direct study of infected tissues (Vago et al., 1996). In human Chagas disease, a different kDNA signature has been demonstrated by this technique for each chronic chagasic patient analyzed (Vago et al., 2000). A high degree of genetic polymorphism has also been observed in the kDNA sequences of *T. cruzi* strains isolated from patients with the same clinical form of the disease (Lages-Silva et al., 2006). Such kDNA minicircle signatures obtained by LSSP-PCR may constitute an important clinical tool for studying the molecular epidemiology of Chagas disease (Vago et al., 2000).

In this study we employed the LSSP-PCR technique to compare the genetic profile of *T. cruzi* kDNA minicircles obtained from the peripheral blood and oesophageal mucosa of the same patient, with or without megaesophagus, from different geographic regions of Brazil, in order to determine a possible association between the two tissues.

2. Materials and methods

2.1. Patients

Our sample was composed of 28 chronic chagasic patients with previous serological tests indicative of Chagas disease, who were submitted to endoscopic examination from August 2003 to December 2005 at the Hospital de Base, São José do Rio Preto, Brazil. Peripheral blood and oesophageal mucosa biopsies (lower third) were collected from each patient. Among the 28 patients there were 18 with megaesophagus or mixed clinical forms (group A) and 10 with an indeterminate or isolated clinical form without megaesophagus (group B). The two groups constituted 16 male and 12 female patients with ages ranging from 45 to 83 years (mean 63.9 ± 11.1) from two main regions of Brazil: the southeast (São Paulo and Paraná) and central west/northeast (Mato Grosso, Minas Gerais and Bahia).

The diagnosis of megaesophagus was established based on clinical data reporting oesophageal obstruction and on manometric and radiological studies. In group A, 10/18 (56%) had only megaesophagus (ME), while 8/18 (44%) had mixed forms [ME, C (cardiopathy) and MCO (megacolon)]. ME was classified into grades I/II (two cases, 11.1%) and III/IV (16 cases, 88.9%), according to the classification of Rezende et al. (1960). In the 10 group B patients (chagasic without megaesophagus) half had the indeterminate (I) form of Chagas disease and the other half had C or MCO. Around 74% of the patients in groups A and B presented discrete-to-severe oesophagitis. Written informed consent was obtained from all patients.

2.2. DNA extraction from blood and oesophageal mucosa

Peripheral blood (7 ml) was collected from 17/18 group A and 8/10 group B patients, immediately mixed with an equal volume of a 6 M guanidine HCl/0.2 M EDTA solution, stored at room temperature for 1 week and subsequently at 4 °C until processed. Before DNA extraction the blood samples were immersed for 15 min in boiling water and, after cooling to room temperature, a 4 ml aliquot was taken from each sample and the DNA extracted once with phenol then twice with chloroform:isoamyl alcohol (24:1) prior to precipitation with two volumes of ethanol in 3 M potassium acetate (pH 6.0). After centrifugation the pellets were re-suspended in 100 μl of ultrapure water and used in concentrated form or in 10-fold dilution for the PCR reactions following Wincker et al. (1994), with modifications.

Oesophageal mucosa biopsies were obtained from all the chagasic patients and exhaustively washed with isotonic saline. For DNA extraction, tissue samples were minced and subjected to the alkaline lysis method described by Vago et al. (1996).

2.3. PCR conditions

Parasite detection in blood and mucosa was performed by specific PCR amplification of the 330 bp fragment corresponding to the four variable regions of the parasite kDNA minicircles. The PCR reactions were performed in a final volume of 25 μl containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 2.0 mM $MgSO_4$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 38 ρmol of the primers S35 and S36, 1.5 U of Taq DNA polymerase (Phoneutria, Belo Horizonte, Minas Gerais, Brazil) and 3 μl of blood or mucosa DNA solution. Thirty-five amplification cycles were carried out under the following conditions: denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min, and extension at 72 °C for 2 min, preceded by an initial denaturation step at 94 °C for 5 min. PCR products were submitted to electrophoresis on a 6% polyacrylamide gel and visualized by silver staining according to Vago et al. (2000), with modifications.

2.4. kDNA signatures

The production of kDNA signatures by LSSP-PCR is a two-step procedure (Vago et al., 2000). The first step consists of specific PCR amplification of the 330 bp fragment of the *T. cruzi* kDNA minicircle. The second step consists of PCR products electrophoresis (330 bp) on a 1.5% agarose gel (1/3 low-melting-point agarose; Promega, Madison, WI, USA) stained with ethidium bromide, with removal of the fragment under ultraviolet light and 10-fold dilution in ultrapure water, to be used as the template for a second amplification step under low stringency conditions (the LSSP-PCR reaction), using only the S35 primer as driver. The LSSP-PCR profiles were visualized by silver staining after elec-