

# Real time PCR strategy for the identification of major lineages of *Trypanosoma cruzi* directly in chronically infected human tissues

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## Abstract

Two evolutionary lineages, called *Trypanosoma cruzi* I and II, have been identified in *T. cruzi*, the etiologic agent of human Chagas disease. Here, we describe a molecular strategy for direct genetic typing of these major groups of *T. cruzi* directly in human tissues. The protocol is based on heminested PCR amplification of the D7 region of the 24Sα ribosomal DNA (rDNA), followed by identification of the products using denaturation curves in real time PCR. The repetitive nature of the gene, and the heminested PCR format insured the high sensitivity necessary to detect the presence of the very scarce *T. cruzi* DNA present in the chronically infected human tissues. There is 80% DNA sequence homology between the two 24Sα rDNA alleles that define the *T. cruzi* I and II groups, sufficient to produce different thermal denaturation curves with melting temperature ( $T_M$ ) values of  $81.7 \pm 0.43$  and  $78.2 \pm 0.33$  °C (mean  $\pm$  SEM). Using this technical approach, we analysed tissue samples (esophagi, hearts and colon) from 25 different patients with the gastrointestinal or cardiac forms of Chagas disease; in all of them we found only the presence of *T. cruzi* II. Previous epidemiological and immunological findings had already led to the idea that chronic human infections occurring in Brazil and Argentina might be primarily due to *T. cruzi* II strains, but all the evidence available had been indirect. Our findings provide definitive proof of this hypothesis and will also allow the establishment of which group of *T. cruzi* is responsible for Chagas disease in other countries.

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**Keywords:** *Trypanosoma cruzi*; rDNA; Real time PCR; Chagas disease

## 1. Introduction

The protozoan *Trypanosoma cruzi* is the causative agent of Chagas disease, a malady that afflicts more than 18 million people in the Americas (Macedo et al., 2004; <http://www.who.int/tdr/publications/tdrnews/news62/chagas.htm>). Following infection of a patient by trypanosomes in hemiptera stools, there is a short acute phase characterised by an abundant parasitemia, but frequently very mild and non-specific symptoms. In this phase the *T. cruzi* can be easily detected in practically all host tissues (Lenzi et al., 1996; Monteon et al., 1996; Tarleton et al., 1997). The infection then proceeds to a chronic phase, with scarce

parasitemia and an unpredictable clinical course that ranges from an absence of symptoms to severe disease with cardiovascular and/or gastrointestinal involvement (Prata, 2001). In this phase of the disease the tissue parasitism is very low and restricted to few anatomical sites such as heart, skeletal muscle, gut or, more rarely, brain (Melo and Brener, 1978; Postan et al., 1984, 1986; Vago et al., 1996a; Lane et al., 1997; Reis et al., 1997; Macedo and Pena, 1998). The ability of the parasite to survive the acute phase and advance into the chronic phase, as well as the distribution of parasites among different host tissues seems to be dependent on both host and parasite factors (Macedo et al., 2004). However, it is generally believed that parasite variability, which has been amply demonstrated in biological, biochemical and molecular analyses, is the most important element (Macedo et al., 1992; Tibayrenc et al., 1993; Souto et al., 1996; Oliveira et al., 1998). On the other hand, many studies have

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tried unsuccessfully to correlate the genetic variability of the parasite with clinical characteristics of the disease (Macedo et al., 2004). One possible explanation for this is the observation that many *T. cruzi* populations are polyclonal. Since most of the techniques used to profile *T. cruzi* require parasite isolation from patient blood and growth in animals or in vitro cultures, they offer ample opportunity for subpopulation selection (Macedo and Pena, 1998). Furthermore, because of possible differential tropism of different *T. cruzi* strains, the clones present in blood and available for laboratory analysis can be very different from those actually causing tissue lesions and most likely associated with the clinical presentation of the patient (Melo and Brener, 1978; Macedo et al., 1992; Macedo and Pena, 1998).

Since 1999, two major lineages named *T. cruzi* I and II have been officially recognised by an international expert committee (Satellite-meeting, 1999). *Trypanosoma cruzi* I strains correspond to the previously defined rDNA group 2, minixenon 2, zymodeme Z1 (Miles et al., 1977) and are primarily associated with sylvatic transmission cycle, while *T. cruzi* II strains, which correspond to rDNA group 1, minixenon 1 and zymodeme Z2 (Miles et al., 1977) are related to the domestic cycle and have been isolated from the blood of chronically infected patients in endemic areas of Argentina, Chile, Bolivia and Brazil. (Satellite-meeting. Recommendations from an International Symposium to commemorate the 90th anniversary of the discovery of Chagas disease, 11–16 April 1999 Rio de Janeiro, Brazil. Mem. Inst. Oswaldo Cruz, 94:429–432.) Indeed, recent immunological evidence has suggested that human infections occurring in the Southern Cone countries are exclusively due to *T. cruzi* II strains (Di Noia et al., 2002), but these findings were based on the host response and not in a direct parasite assay. On the other hand, there are epidemiological indications that chronic human infections in northern South America and Central America may be caused by *T. cruzi* I (Miles et al., 1981).

We have shown that it is possible to obtain a genetic profile kinetoplast DNA (kDNA) signatures of *T. cruzi* parasites that chronically infect human tissues by the technique of Low-stringency Single Specific Primer-PCR (LSSP-PCR) (Vago et al., 1996b, 2000). Although highly sensitive, this method is not capable of identifying to which major lineage the *T. cruzi* present in the tissue belongs. We wish to describe a new strategy capable of achieving this which is based on heminested PCR amplification of the D7 region of the 24S $\alpha$  rRNA gene, followed by identification of the products using denaturation curves in real time PCR. The repetitive nature of the gene (approximately 110 copies per parasite) and the heminested PCR format insure the high sensitivity necessary to detect the presence of the very scarce *T. cruzi* DNA present in the chronically infected human tissues. Two 24S $\alpha$  rDNA alleles of 110 and 125 bp define the *T. cruzi* I and II groups, respectively. There is 80% DNA sequence homology between the two types of

amplicons, sufficient to produce different thermal denaturation curves with melting temperatures ( $T_M$ ) of  $81.7 \pm 0.43$  and  $78.2 \pm 0.33$  °C (mean  $\pm$  SEM), respectively, as determined using DNA from several strains previously classified as belonging to *T. cruzi* I or II.

## 2. Material and methods

### 2.1. Parasites

Two *T. cruzi* populations, originally isolated from the blood of patients with distinct forms of Chagas disease, were used: JG (*T. cruzi* II, D7 rDNA group 1) and Col1.7G2 (*T. cruzi* I, D7 rDNA group 2). The JG strain was isolated from a patient from Minas Gerais, Brazil with megaesophagus and was typed as a monoclonal strain using eight different microsatellite loci (Oliveira et al., 1998). Col1.7G2 is a clone obtained by serial dilution from the Colombian strain, which was originally isolated from the blood of a chronically infected patient with cardiac disease (Federici et al., 1964).

### 2.2. Infected mice

Inbred male mice (BALB/c) were inoculated intraperitoneally with a mixture of JG strain and Col1.7G2 clone (50+50 trypomastigotes). Infected animals and age-matched controls were sacrificed in the chronic phase defined as 6 months of infection, according to the code of ethics of the COBEA (Colégio Brasileiro de Experimentação Animal). Tissue samples from the heart and rectum were washed exhaustively in isotonic saline and stored in ethanol at  $-20$  °C.

### 2.3. Patients

Twenty-eight tissue samples obtained from 24 chagasic patients resident in different cities of Minas Gerais and one from Goiás, Brazil were analysed. As negative controls we analysed tissue samples from three non-chagasic patients (Table 1). Samples of esophagi and colon were obtained by surgical procedures. Heart samples were obtained from autopsies of patients who died from complications of Chagas disease or, in one case, AIDS. This study fulfilled all the criteria required by the Medical Code of Ethics and the Helsinki II statement and was approved by two independent Ethical Committees (Comitê de Ética da Faculdade do Triângulo Mineiro and Comitê de Ética da Universidade Federal de Minas Gerais). Human tissue slices were rinsed in sterile saline and stored in ethanol at  $-20$  °C for DNA extraction.

### 2.4. Parasite and tissue DNA extractions

*T. cruzi* epimastigotes were grown in liver infusion tryptose (LIT) medium containing 10% calf serum at

Table 1  
Patient tissues samples

Sample	Tissue sample	Residence	Clinical form
188	Heart (LV)	Araxá, MG	C(I)
A29A	Heart	MG	C(IV)
A30A	Heart	MG	C(VI)
A332	Heart	MG	C(II)
A36B	Heart	MG	C(IV)
A37A	Heart	MG	C(III); MO
AMN	Heart (RA)	Brumadinho, MG	C(IV)
AMN	Heart (LV)	Brumadinho, MG	C(IV)
DF	Heart (RA)	Sto Antônio de Pirapitinga, MG	C(IV)
DF	Heart (LV)	Sto Antônio de Pirapitinga, MG	C(IV)
JP	Heart	Capelinha, MG	C(IV)
1023	Colon	Carneirinhos, MG	?
E4A	Esophagus	MG	MO
E8A	Esophagus	MG	MO(IV)
E11A	Esophagus	MG	C(IV)
E12C	Esophagus	MG	MO(II); C(II)
24	Esophagus	Araxá, MG	MO(IV)
179	Esophagus	Uberaba, MG	C(II); MO(IV); MC (I)
188	Esophagus	Araxá, MG	C(I)
213	Esophagus	Uberaba, MG	MO(IV); MC(I)
260	Esophagus	Santa Rosa, MG	C(II); MO(II)
504	Esophagus	Salinas, MG	C(II); MO(IV); MC(I)
511	Esophagus	Córrego Danta, MG	MO(IV); C(II)
523	Esophagus	Carmópolis de Minas, MG	CII-MEIV
690	Esophagus	Guarani, GO	?
927	Esophagus	Santa Juliana, MG	C(III); MO(III)
961	Esophagus	Frutal, MG	MO(III)
1086	Esophagus	MG	?
<i>Non-chagasic patients (negative controls)</i>			
JLG	Esophagus	Uberlândia, MG	Esophagus neoplasia
MLO	Esophagus	MG	Esophagus neoplasia
MAC03	Colon	Perú	Idiopathic megacolon

RA, right atrium; LV, left ventricle. Clinical forms: C, cardiopathy; MO, megaesophagus; MC, megacolon. Severity levels: I, low severity to IV high severity.

27–28 °C. The parasites were harvested, rinsed in sterile saline and stored at –70 °C until the standard DNA extraction with phenol/chloroform (Andrade et al., 1999).

Fragments of human or mice tissues were rinsed in sterile saline and stored in absolute ethanol at –20 °C until used. For DNA extraction the tissue fragments were exhaustively sliced and submitted to alkaline lysis by treating them with 50 mM NaOH for 10 min at 100 °C followed by neutralisation with 130 mM Tris–HCl (pH 7.0). Supernatant aliquots were used directly in the PCR reaction or alternatively after 10-fold dilution in twice distilled water.

### 2.5. PCR amplification of *T. cruzi* kDNA

For all analysed tissues the presence of *T. cruzi* was initially evaluated by amplification of variable regions of minicircles (kDNA) by using the S35 (5'-AAATAATG-TACGGGGAGATGCATGA-3') and the S36 (5'-GGG TTCGATTGGGGTTGGTGT-3') primers as described early (Vago et al., 1996b). PCR products (~330 bp) were visualised in 6% polyacrylamide gels after silver staining (Santos et al., 1993).

### 2.6. Characterisation of the parasites by heminested PCR amplification and real time PCR

For amplification and typing, 5 ng of parasite DNA or 5 µl of the product of the alkaline lysis obtained from human tissue samples were used as template; a first round with 0.25 µM of each primer (D75=5'-CAGATCTTGG TTGGCGTAG-3' and D72=5'-TTTTCAGAATGGCC-GAACAGT-3'), 200 mM of each dNTP and 0.75 units of *Taq* polymerase (Promega) in 50 mM KCl, 10 mM Tris–HCl, a volume of 20 µl reaction. Thirty-five amplification cycles were performed with an annealing temperature at 60 °C, extension at 72 °C and denaturation at 94 °C, each one for 45 s in an Eppendorf MasterCycler Gradient thermocycler. Two microliters of the PCR products were used as template in the second PCR round performed in a real time PCR apparatus (ABI7900—Applied Biosystems). The reactions were done in SYBR® Green PCR Master Mix (Applied Biosystems) with 0.25 µM each of D71 (5'-AAGGTGCGTCGACAGTGTGG-3') and D72 primers. After an initial step of 50 °C for 5 min (to activate the ampUNG that avoids cross contaminations) and 95 °C for 10 min (to inactivate the ampUNG and activate *Taq*GOLD), 40 cycles of 60 and 95 °C for 1 min each were carried out. Finally, the PCR products were slowly warmed up from 60 to 95 °C and the rDNA amplicon melting point was determined using the dissociation curve tool available in the ABI 7900.

## 3. Results

### 3.1. Parasite DNA analyses

The possibility of distinguishing the two D7 alleles (110 and 125 bp) by analysis of their melting temperature were

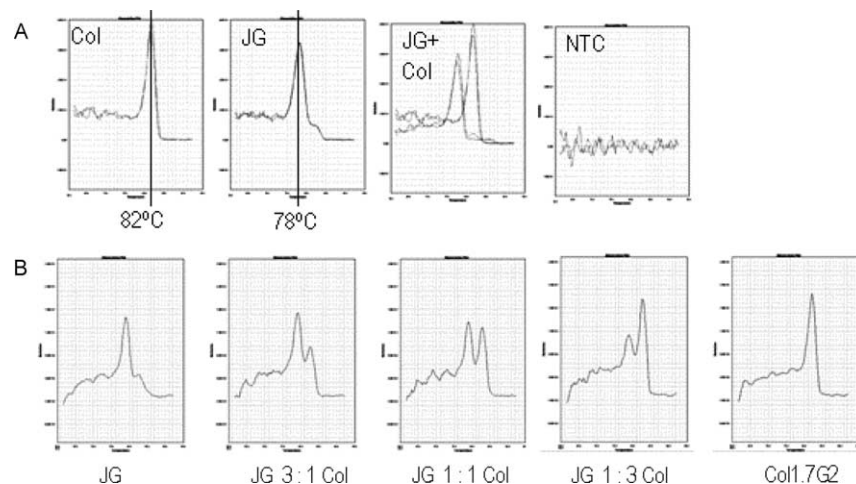


Fig. 1. Melting curves obtained for the amplicons obtained by heminested amplification of D7 domain from (A) 100 fg of JG or/and Col DNA and no template control (NTC) and (B) different proportions of mixture of JG and Col 1.7G2 DNA.

initially investigated by using DNA extracted from epimastigote forms of cultured Col1.7G2 and JG *T. cruzi* strains. The two strains presented  $T_M$  of 81.5 and 78.2 °C, respectively, easily distinguishable in the real time apparatus (Fig. 1A). By associating the heminested PCR format with the real time PCR we could detect amplicons in as little as 10 fg of parasite DNA, which is much less than the amount of DNA present in a single parasite (data not shown).

In order to test if the discrimination was universal, we typed two replicates using 1 ng of total DNA from seven different strains of *T. cruzi* I and six of *T. cruzi* II (Table 2). The average value of *T. cruzi* I was  $81.7 \pm 0.43$  °C and *T. cruzi* II was  $78.2 \pm 0.33$  °C. The data were analysed using nested analysis of variance. The difference between the *T. cruzi* I and II groups was highly significant ( $F=35.7$ , 1 df,  $P<0.001$ ). Interestingly the variation between strains within *T. cruzi* groups was also significant ( $F=12.7$ , 12 df,  $P<0.001$ ).

When DNA from JG and Col1.7G2 were mixed in different proportions (Fig. 1B), this was reflected in the relative height of the  $T_M$  peaks, suggesting that this measure (or the area under the peak) could be used as a quantitative estimative of the proportion of each population in the mixture.

### 3.2. Mice tissue analyses

The capacity of detecting and discriminating between the two D7 alleles was further verified by analyzing doubly infected BALB/c heart and rectum tissues (Fig. 2). We could clearly identify the predominance of the *T. cruzi* I allele (JG strain) in the hearts and the preponderance of the *T. cruzi* I allele (Col1.7G2) in the recta of the same mice infected with both *T. cruzi* populations. These results matched perfectly our previous results using LSSP/PCR kDNA signatures (boxes in Fig. 2; Andrade et al., 1999).

### 3.3. Human tissue analyses

Human heart, colon and esophageal tissues obtained from chagasic and non-chagasic patients were initially investigated for the presence of *T. cruzi* DNA using amplification of minicircle sequences as previously described (Vago et al., 1996a; data not shown). From the 28 investigated tissue samples positive for kDNA amplicons 27 were also positive in the heminested rDNA real time PCR, thus permitting the classification of the rDNA allele. In all cases only the allele with  $T_M$   $78.2 \pm 0.33$  °C, corresponding to the *T. cruzi* II major lineage, was seen (Fig. 3).

Table 2

Determination of the melting point of D7 amplicons from seven strains of *Trypanosoma cruzi* I and seven strains of *T. cruzi* II

<i>T. cruzi</i> group	I							II						
Strain	1004	Cuíca	D7	Gambá c11	GLT 600	RbVI	125F	Gilmar	GOCH	JM	MCS	MPD	Per 18-5	580
Replicate 1	81.5	81.6	82.1	81	81.7	82.2	81.5	78.3	77.4	78.6	78.1	78.3	78.3	78.0
Replicate 2	81.5	81.2	82.5	81.1	81.6	82.3	81.4	78.4	77.6	78.5	78.5	78.3	78.2	77.6

All values in °C.



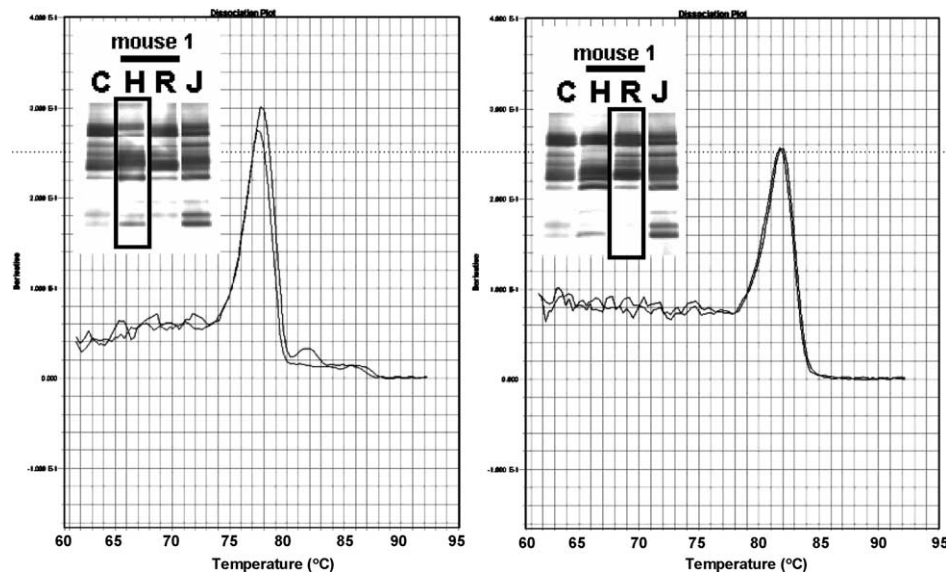


Fig. 2. D7 domain melting curves obtained from mice tissues artificially infected with JG and Col1.7G2. Samples were previously analysed by LSSP/PCR (boxes at left of each curve) in comparison with the result of rDNA analyses. LSSP/PCR profiles obtained from: (J) JG, (C) Col1.7G2, (H) heart and (R) rectum DNA.

#### 4. Discussion

In this work we describe a heminested PCR strategy for amplification of the D7 polymorphic region of 24S $\alpha$  rRNA gene and assay of the parasite major lineages directly in chronically infected human and animal tissues. Differences in the CG content (Kawashita et al., 2001) and therefore in the  $T_M$  of the D7 alleles constitute the base of the present approach. The  $T_M$  values are statistically different for each D7 allele and allow us to determine the parasite major lineage independent of the specific strain utilised (Table 2). Moreover, nested ANOVA revealed significant differences between the *T. cruzi* I and the II groups. This is due to minor sequence differences within the sequence of the rDNA D7 region between the strains. This means that, at least in some cases, it may be possible to estimate the identity of a strain just by determining the  $T_M$  of the D7 amplicon.

To investigate the possibility of using the present methodology to profile the parasites directly in the chronically infected tissues, we initially analysed heart and recta of BALB/c mice double infected with mixtures of JG and Col1.7G2 tripomastigotes. Andrade et al. (1999), using kDNA signatures obtained by the LSSP-PCR technique had already identified a differential tropism in these animals. After 6 months of infection, the JG strain was found in the heart while Col1.7G2 clone was predominantly found in the recta of the same animals (Andrade et al., 1999). The LSSP-PCR methodology, although very valuable in identifying the differential tissue tropism, is useful mainly if the original strains' profiles are known and gives little or no phylogenetic

information. Analysis of the same doubly infected mice tissues by D7 rDNA domain demonstrated a perfect correlation between these results. In all cases where detected the presence of Col1.7G2 LSSP/PCR profile, the rDNA allele amplified had a  $T_M$  of 81.5 °C, characteristic of the clone Col1.7G2. When analyzing tissues that had showed a JG LSSP/PCR profile, the amplified rDNA allele amplified presented a  $T_M$  of 78.2 °C typical of this strain.

Typing of the D7 rDNA allele directly in chronically infected tissues were performed on 28 human tissues from 25 patients from endemic areas in the states of Minas Gerais and Goiás in Brazil. All these samples had been previously tested for the presence of *T. cruzi* DNA by amplification of kDNA (data not shown). From them 27 samples amplified the D7 rDNA showing that rDNA amplification using the heminested protocol may have sensitivity comparable to kDNA amplification, with the advantage of allowing lineage identification. All of the Brazilian patient samples analysed displayed only the rDNA allele with  $T_M$  in the vicinity of 78 °C, typical of *T. cruzi* II strains.

Previous indirect epidemiological (Miles et al., 1981; Vago et al., 1996a; Macedo et al., 2004) and immunological (Di Noia et al., 2002) studies had already suggested that *T. cruzi* II strains were responsible for Chagas disease in Argentina and Brazil. However, the present work is the first direct demonstration that *T. cruzi* II is indeed the causative agent of the tissue lesions of Chagas disease in Brazil. This demonstration has pathogenetic implications and also might prove to have therapeutic importance. The availability of our new methodology for direct typing of *T. cruzi* in human

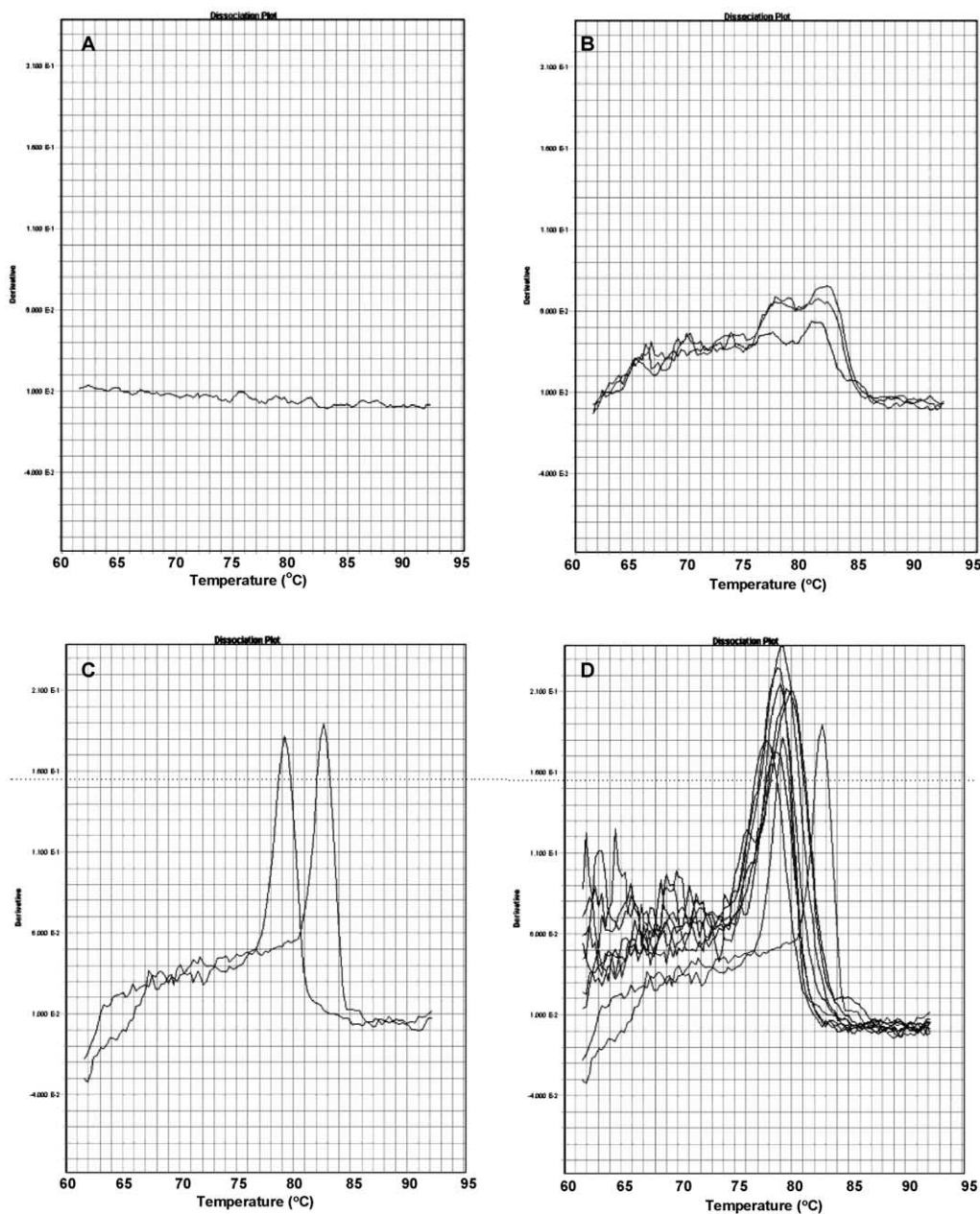


Fig. 3. D7 domain melting curves obtained from: (A) no template control; (B) three DNA samples from non-Chagasic patients; (C) amplifications of JG and Col1.7G2 DNA and (D) superimposition of curves obtained with seven DNA samples from hearts and esophagi of Chagasic patients.

tissues will allow the establishment of which group of *T. cruzi* is responsible for Chagas disease in other countries of South America, Central America and in Mexico.

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