Acta Tropica 133 (2014) 98–109

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Circulating *Trypanosoma cruzi* populations differ from those found in the tissues of the same host during acute experimental infection

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ARTICLE INFO

Article history:
Received 10 July 2013
Received in revised form
20 December 2013
Accepted 11 February 2014
Available online 19 February 2014

Keywords:
Acute phase of Chagas disease
natural isolates
tissue distribution
Trypanosoma cruzi lineages or DTUs

ABSTRACT

We evaluated the presence and distribution of two *Trypanosoma cruzi* natural isolates in blood, heart, skeletal muscle, liver, and spleen tissues in the acute phase of the experimental infection (35 days post-infection) in order to determine if the populations present in blood were different to those found in the tissues of the same host. Thirty mice were infected with 50 forms of each isolate or with a combination of them. Presence and molecular characterization of the parasites in the host tissues were determined by specific PCR. Cardiac and skeletal muscle alterations were analyzed by histological studies. *T. cruzi* variability in the host tissues was analyzed through RFLP studies. Both isolates used consisted of a mixture of two *T. cruzi* lineages. Specific PCRs were positive for most of the samples from the 3 groups analyzed. Cardiac and skeletal muscle sections from the groups infected with one isolate presented mild to moderate inflammatory infiltrates; the group infected with both isolates showed severe inflammatory infiltrates and the presence of amastigote nests in both tissues. Different parasite populations were found in circulation and in the tissues from the same host. These results are important for patients with high probability of mixed infections in endemic areas and contribute to the knowledge of parasite/host interactions.

1. Introduction

Chagas disease is an infection caused by the protozoan parasite *Trypanosoma cruzi* that affects between 10 and 18 million people concentrated in the poorest rural and urban areas of Latin America. The infection represents one of the most serious public health problems in the region (de Lana and Tafuri, 2000; WHO, 2007) where 75 to 90 million people are exposed to *T. cruzi* (Coura, 2007); the infection is also becoming a health problem in nonendemic countries (USA and European countries) due to migration (Schmunis and Yadon, 2010; Pereira Nunes et al., 2013).

Chagas disease presents a variable clinical course, including an acute or initial phase with probable fever, adenomegaly, unilateral conjunctivitis (Romaña's sign), myocarditis, and meningoencephalitis; this initial phase can be fatal in upto 10% of severe cases, with higher mortality in children under three years of age due to meningo-encephalitis (Coura, 2007). The acute phase of Chagas disease is characterized by the presence of circulating parasites in the host. After this phase, the infection evolves to an asymptomatic and silent chronic latency period of 10 to 15 years, referred to as the indeterminate form (Coura, 2007), after which about 27% of infected individuals develop cardiac symptoms that can lead to sudden death, 6% develop digestive damage (megacolon and/or megaesophagus), and 3% can present alterations in the peripheral nervous system (Carlier and Truyens, 2010; WHO, 2007; Pereira Nunes et al., 2013). The clinical features of Chagas disease range therefore from asymptomatic cases to severe chronic cardiovascular and/or gastrointestinal involvement. Due to biological polymorphism, different clones in a lineage can present tropism for different tissues, becoming a determinant factor for the disease' clinical course due to the clonal repertoire of the infecting lineage and its specific tropisms (Macedo and Pena, 1998). The events that take place in the acute phase may influence the course of the later infection.

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http://dx.doi.org/10.1016/j.actatropica.2014.02.010
0001-706X/

The most interesting challenge to understand the pathophysiology of Chagas disease still lies in the complex interaction between *T. cruzi* and the mammalian host. The genetic variability of both the parasite and the host can determine the clinical evolution of the infection (Macedo and Pena, 1998; Freitas et al., 2009). *T. cruzi* strains are complex multiclonal populations that differ in their genetic and biological characteristics (virulence, growth rate, pathogenicity, tissue tropism, and sensitivity to antitrypanocidal drugs among others) and in their behavior in the vertebrate host (Macedo and Pena, 1998). Based on different molecular markers and biological features, *T. cruzi* is currently grouped into six genetic lineages or Discrete Typing Units (DTUs), previously named TcI, TcIIa, IIb, IIc, IId, and IIe (Brisse et al., 2000; 2001) but recently revised by a broad consensus to TcI, TcIV, TcII, TcIII, TcV, and TcVI, respectively (Zingales et al., 2009; 2012).

Natural infections are usually constituted by multiple clones with differential tropism to host tissues, which can vary throughout the infection (Andrade and Magalhâes, 1997; Andrade et al., 2010; Mejia and Triana, 2005; Roellig et al., 2010; Vago et al., 2000). The distribution of these clones in the host could determine the clinical features and the evolution of the infection in patients and experimental models. Several studies have confirmed the high incidence of multiple infections (with more than one parasite strain or isolate) in humans (Mantilla et al., 2010; Solari et al., 2001) and vectors (Bosseno et al., 2000), which highlights the relevance of this parameter for the evolution of the infection.

In the present study we evaluated the presence and distribution of two *T. cruzi* natural isolates (both obtained from congenitally infected patients) in blood, cardiac and skeletal muscles, liver, and spleen from mice infected with either a single isolate or a combination of them, in the acute phase of the experimental infection in order to determine if the populations present in blood were different from those found in the tissues of the same host.

2. Materials and Methods

2.1. Parasites and infection scheme

Blood trypomastigote forms from two *Trypanosoma cruzi* isolates obtained from congenitally infected patients were used to inoculate 30 outbred Albino Swiss mice (2 months old males and females, each weighting 30 ± 1 g). Lucky (Triquell et al., 2009) and Casibla isolates of *T. cruzi* were originally obtained from umbilical cord blood of congenitally infected newborn child from endemic area in Argentina. Both parasite isolates used have been maintained in the laboratory by successive infections of new mice every 15 days. Mice had been kept in controlled housing conditions (12 h light period, 25 °C, food and water *ad libitum*) and the experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U. S. National Institute of Health, publication N° 85-23, (revised 1996) and had been approved by the Institutional Committee for the Care and Use of Laboratory Animals from the Faculty of Medicine, National University of Córdoba, Argentina.

Each mouse was inoculated (by intraperitoneal injection) with each isolate according to the following groups:

- **Group 1**: 10 mice infected with 50 bloodstream trypomastigote forms of isolate 1 (Casibla).
- **Group 2**: 10 mice infected with 50 bloodstream trypomastigote forms of isolate 2 (Lucky).
- **Group 3**: 10 mice infected with 50 bloodstream trypomastigote forms of isolate 1 and 50 bloodstream trypomastigote forms of isolate 2. The purpose to include this doubled-infected group was to verify if the parasite distribution was affected by the presence of a greater amount of isolates; the parasite inoculum therefore was chosen to be comparable with either of the single-infected groups.

The number of parasites per milliliter of blood was estimated in a Neubauer hemocytometer and has been previously found to be enough to reproduce the acute phase of the infection in the murine model (Bustamante et al., 2003; Lo Presti et al., 2006). To verify the parasite presence and differential distribution in host tissues (blood, heart, skeletal muscle, liver, and spleen), infected animals were sacrificed in the acute phase of the experimental infection (35 days postinfection –d.p.i.) (Bustamante et al., 2003; Lo Presti et al., 2006). A molecular characterization of the isolates was also performed.

2.2. Parasitemia and survival

Circulating parasite levels were determined in a Neubauer hemocytometer using blood samples obtained from the tail once a week, beginning 7 days after the infection until day 35 postinfection (p.i.), as previously described for similar models (Bustamante et al., 2003; Lo Presti et al., 2006). Survival of the different groups was monitored daily; all the animals that survived to day 35 p.i. in the different groups were used for the rest of the experiments.

2.3. Cardiac and skeletal muscle histological studies to characterize the infection with these particular isolates

Mice were sacrificed by decapitation, using Ketamine ClH (Ketalar®, Parke Davis, Warner Lambert Co, USA) anesthesia (10 mg/Kg). The hearts (n = 3 for each group) were removed, fixed in buffered 10% formaldehyde (pH 7.0), and embedded in paraffin. Each heart was cut horizontally into 5 μm sections and stained with Hematoxiline-Eosine. A total of 9 slices from each group were analyzed using 4X, 10X, 20X, and 40X objectives. The same procedures were followed for skeletal muscle samples (n = 3 for each group) obtained from the right posterior leg of the mice from the different groups. The area covered by inflammatory infiltrates in each sample (in 4X images) was quantified using AxioVision 4.8 program (Carl Zeiss Imaging Solutions). Images were also examined for the presence of *T. cruzi* amastigote nests.

2.4. DNA extraction from blood and tissue samples

Blood samples from each infected mice (1 mL) were mixed with an equal volume of guanidine hydrochloride 6 M/EDTA 0.2 M (Avila et al., 1991); cardiac, skeletal muscle, liver, and spleen samples were lysed with CTAB (cethyl-trymethylamonium bromide) (Levitan and Grosberg, 1993). DNA was extracted from 200 μL of the blood/guanidine mixture, using conventional phenol:chlorophorm:isoamylic techniques (Lachaud et al., 2001), precipitated with ethanol and re-suspended in sterile nuclease free water. The samples were conserved at – 20 °C (Wincker et al., 1994) until used for the amplification of the parasite DNA by the Polymerase Chain Reaction (PCR).

2.5. T. cruzi detection and molecular characterization

T. cruzi detection in each sample was performed by the amplification of a 188 bp nuclear fragment of the parasite DNA using two specific primers: **TCZ-1** (5′CGA GCT CTT GCC CAC ACG GGT GCT 3′) and **TCZ-2** (5′CCT CCA AGC AGC GGA TAG TTC AGG 3′) (Virreira et al., 2003).

Molecular characterization of *T. cruzi* was carried out using three molecular markers that allow the identification of the six *T. cruzi* lineages (following the algorithm described in figure 1), as

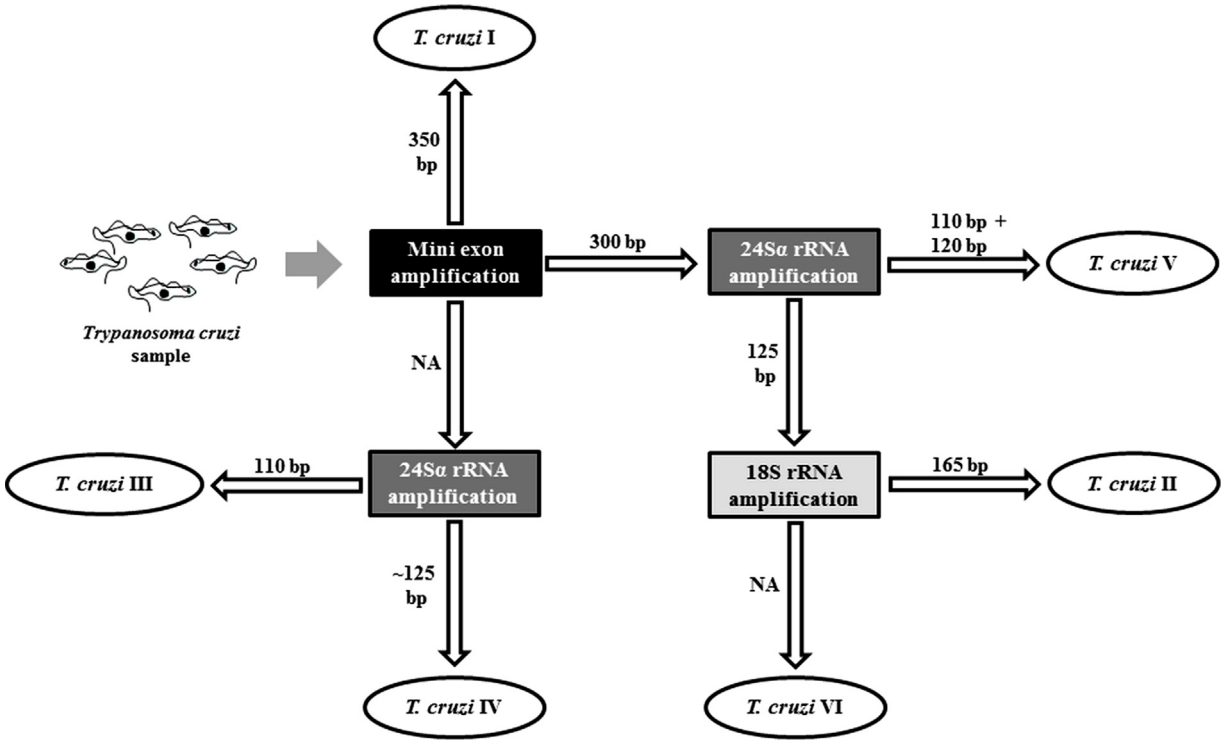

Figure 1. Algorithm used for the *T. cruzi* lineage classification using three molecular markers (modified from Ramírez *et al*, 2010). NA: no amplification; bp: base pairs.

previously described by several authors (Brisse et al., 2001; Ramírez et al., 2010; Steindel et al., 2008; Velazquez et al., 2008): the intergenic region of the nontranscribed mini-exon gene using primers **TC** (5′CCC CCC TCC CAG GCC ACA CTG 3′), **TC1** (5′GTG TCC GCC ACC TCC TTCGGG CC 3′), and **TC2** (5′CCT GCA GGC ACA CGT GTG TGT G 3′) (Souto et al., 1996), the D7 divergent domain of the 24Sα rRNA using primers **D71** (5′AAG GTG CGT CGA CAG TGT GG 3′) and **D72** (5′TTT TCA GAA TGG CCG AAC AGT 3′) (Souto et al., 1996), and the variable domain of the 18S rRNA sequence using the primers **V1** (5′CAA GCG GCT GGG TGG TTA TTC CA 3′) and **V2** (5′TTG AGG GAA GGC ATG ACA CAT GT 3′) (Clark and Pung, 1994).

In order to confirm the *T. cruzi* lineage present in each tissue, a second set of molecular markers was used as described by Cosentino and Agüero (2012): the amplification followed by the digestion of the TcSC5D amplicon with a mixture of Sph I and Hpa I restriction enzymes, which results in unique patterns for all DTUs, except for DTUs TcV and TcVI, that cannot be differentiated, and the amplification followed by the digestion of the TcMK amplicon with Xho I to differentiate TcV from TcVI samples. A schematic representation of the expected restriction fragments in each case can be observed in Figure 2. A fragment of 832 bp of the TcSC5D gene was amplified using primers **TcSC5D-fwd** (5′GGA CGT GGC GTT TGA TTT AT 3′) and **TcSC5D-rev** (5′ TCC CAT CTT CTT CGT TGA CT 3′), and a fragment of 537 bp of the TcMK gene was amplified using **TcMK-Fw** (5′ TTT TTG CAT GTC ATT TTG G 3′) and **TcMK-Rv** (5′ AGC GGT CTT GTA ATG AGC AC 3′). Restriction enzymes (Hpa I, Sph I, and Xho I – New England Biolabs, Inc.) were used as suggested by the enzyme's manufacturer (37 °C for 1 h); in every case the reaction was ended with a stop solution (50% glycerol, 50 mM EDTA pH 8.0, and 0.05% bromophenol blue) after incubation time had finished.

The quality of the DNA samples was verified by the amplification of a 289 bp constitutive gene from the host (β-actin), using the corresponding primers: **β-act-F** (5′CGG AAC CGC TCA TTG CC 3′) and **β-act-R** (5′AAC CAC ACT GTG CCC ATC TA 3′).

Reaction mixtures composition and amplification conditions for each PCR are described in Tables 1 and 2, respectively. For all the molecular markers, PCR products were separated by electrophoresis in a 2.5% agarose gel stained with ethidium bromide and examined under UV light. Each reaction was performed in triplicate.

2.6. Differential tissue distribution

In order to further characterize the differential tissue distribution of the parasite in the host's tissues (to verify if there was even greater variability within the parasite lineages/populations present in tissues compared to those found in circulation), restriction fragment length polymorphism (RFLP) studies were performed using the amplified mini-exon region (300 pb) and five restriction enzymes (Bce AI, Dra III, Fsp I, Rsa I, and Bsm AI –New England Biolabs, Inc.). Digestion conditions suggested by the enzyme's manufacturer were followed. The obtained fragments were separated by electrophoresis in 2.5% agarose gels stained with ethidium bromide and examined under UV light.

2.7. Data analysis

Parasitemias were compared by multivariate analysis of variance using Hotelling test; survival was studied using Kaplan–Meier Survival test; percentages of histological abnormalities were analyzed using Pearson Chi-square test. PCR results for a given sample were considered negative after three repetitive negative reactions; one positive reaction was considered enough for a positive result.

T. cruzi lineage classification was performed following Ramirez and collaborators (Ramírez et al., 2010) algorithm, modified for three molecular markers (figure 1) and Cosentino and Agüero (2012) typing assay.

Proportions of samples with different RFLP patterns in tissues to those found in blood of the same animal were compared by Pearson Chi-square test. The tissue samples that could present partially digested fragments were considered as such and not counted as different from the blood samples from the same animal.

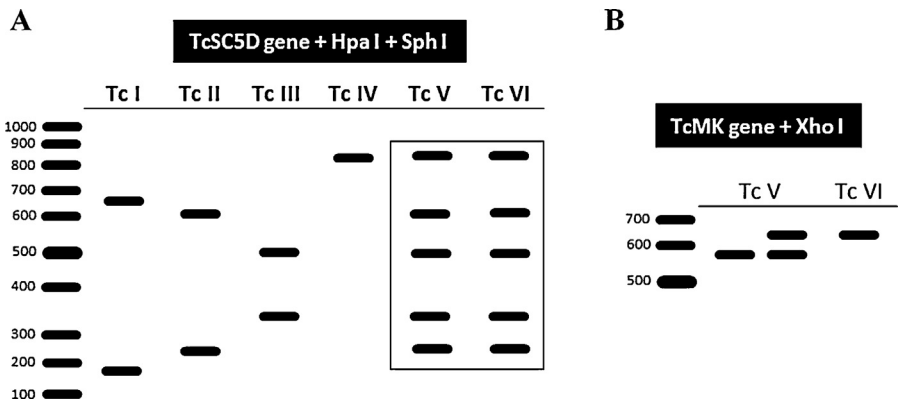

Figure 2. Schematic representation on the *T. cruzi* lineage classification method using two molecular markers (adapted from Cosentino and Agüero, 2012): A) expected restriction fragments after digestion of the TcSC5D amplicon with a mixture of Sph I and Hpa I; B) expected restriction fragments after digestion of the TcMK amplicon with Xho I to differentiate Tc V from Tc VI samples.

Table 1
Reaction mixtures composition for the amplification of the different molecular markers.

Molecular markers	Tris-Cl (pH 8.4)	KCl	Cl_2Mg	dNTP	Taq	Primers	Sample DNA	Final volume
TCZ	10 mM	50 mM	1.5 mM	200 μM	0.5 U	25 ρmols	~ 2.5 ηg	25 μL
Mini-exon	10 mM	50 mM	1.75 mM	200 μM	1 U (HS)	25 ρmols	~ 5 ηg	50 μL
24Sα rRNA	10 mM	50 mM	2 mM	200 μM	1 U	20 ρmols	~ 4 ηg	50 μL
18S rRNA	10 mM	50 mM	2 mM	200 μM	1 U (HS)	20 ρmols	~ 4 ηg	50 μL
TcSC5D	10 mM	50 mM	1.6 mM	200 μM	1.5 U	10 ρmols	~ 5 ηg	25 μL
TcMK	10 mM	50 mM	1.6 mM	200 μM	2 U	10 ρmols	~ 5 ηg	25 μL
β-actin	10 mM	50 mM	1.5 mM	200 μM	0.5 U	10 ρmols	~ 2.5 ηg	25 μL

HS: Hot start Taq Polymerase.

For every case, differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Parasitemia and survival

Blood parasite levels were positive in all the studied groups from day 7 p.i. The groups infected with one isolate (groups 1 and 2) presented a peak in their parasitemia levels between days 21 (**group 1:** 242.79 ± 41.84 parasites per microliter) and 28 p.i. (**group 2:** 835.40 ± 99.53 parasites per microliter), respectively; the maximum circulating parasite levels in the group infected with both isolates together (**group 3**), were present from day 14 (356.41 ± 130.45 parasites per microliter) to day 28 p.i. (329.49 ± 23.63 parasites per microliter). Parasite levels in group 2 were significantly higher than the other groups ($P < 0.05$); group 3 presented intermediate levels between groups 1 and 2 ($P < 0.05$).

No differences were found between the groups when comparing the survival rates observed by day 35 p.i. (group1: 27%; group 2: 28%; group 3: 29%).

3.2. Histological studies

Figures 3, 4 and 5 show representative cardiac and skeletal muscle areas from groups 1, 2, and 3, respectively, used to characterize the infection with these particular natural isolates.

The cardiac samples from **group 1** presented mild to moderate inflammatory infiltrates, with an average of 2.64% of the heart covered by inflammation; no amastigote nests were found in these samples (Figure 3 a, b, and c). The skeletal muscle sections from the same group also presented inflammatory infiltrates but of a higher intensity (Figure 3 d, e, and f); the area covered by infiltrates in this case was of an average of 9.61%. These sections also showed the presence of amastigote nests in the muscle fibers.

The cardiac samples from **group 2** presented moderate to severe inflammatory infiltrates, covering an average area of 3.32% of the total area of the sample (Figure 4 a and b); one animal from this group also presented amastigote nests in the cardiac fibers (Figure 4 c). The inflammatory infiltrates in the skeletal muscle sections covered a greater area than in the hearts from the same group (15.16%) (Figure 4 d – i). Additionally, several amastigote nests were found in the skeletal muscle sections from 2 out of the 3 mice analyzed (Figure 4 g, h, and i).

Table 2
PCR conditions for the amplification of the different molecular markers.

Molecular markers	Initial denaturation step	Number of cycles	Each cycle consisted of:			Final extension step
			Denaturation	Annealing	Extension	
TCZ	4 min; 95 °C	40	30 sec; 95 °C	30 sec; 60 °C	30 sec; 72 °C	5 min; 72 °C
Mini-exon	2 min; 94 °C	30	30sec; 94 °C	30 sec; 55 °C	30 sec; 72 °C	7 min; 72 °C
24Sα rRNA	1 min; 94 °C	30	30 sec; 94 °C	30 sec; 60 °C	30 sec; 72 °C	7 min; 72 °C
18S rRNA	2 min; 94 °C	30	30 sec; 94 °C	30 sec; 50 °C	30 sec; 72 °C	7 min; 72 °C
TcSC5D	4.5 min; 94 °C	35	30 sec; 94 °C	30 sec; 58 °C	30 sec; 72 °C	5 min; 72 °C
TcMK	4.5 min; 94 °C	35	30 sec; 94 °C	30 sec; 58 °C	30 sec; 72 °C	5 min; 72 °C
β-actin	4 min; 95 °C	30	30 sec; 95 °C	30 sec; 60 °C	30 sec; 72 °C	5 min; 72 °C

In all cases, amplifications were performed on DNA Thermal Cycler (Ivema T-18).

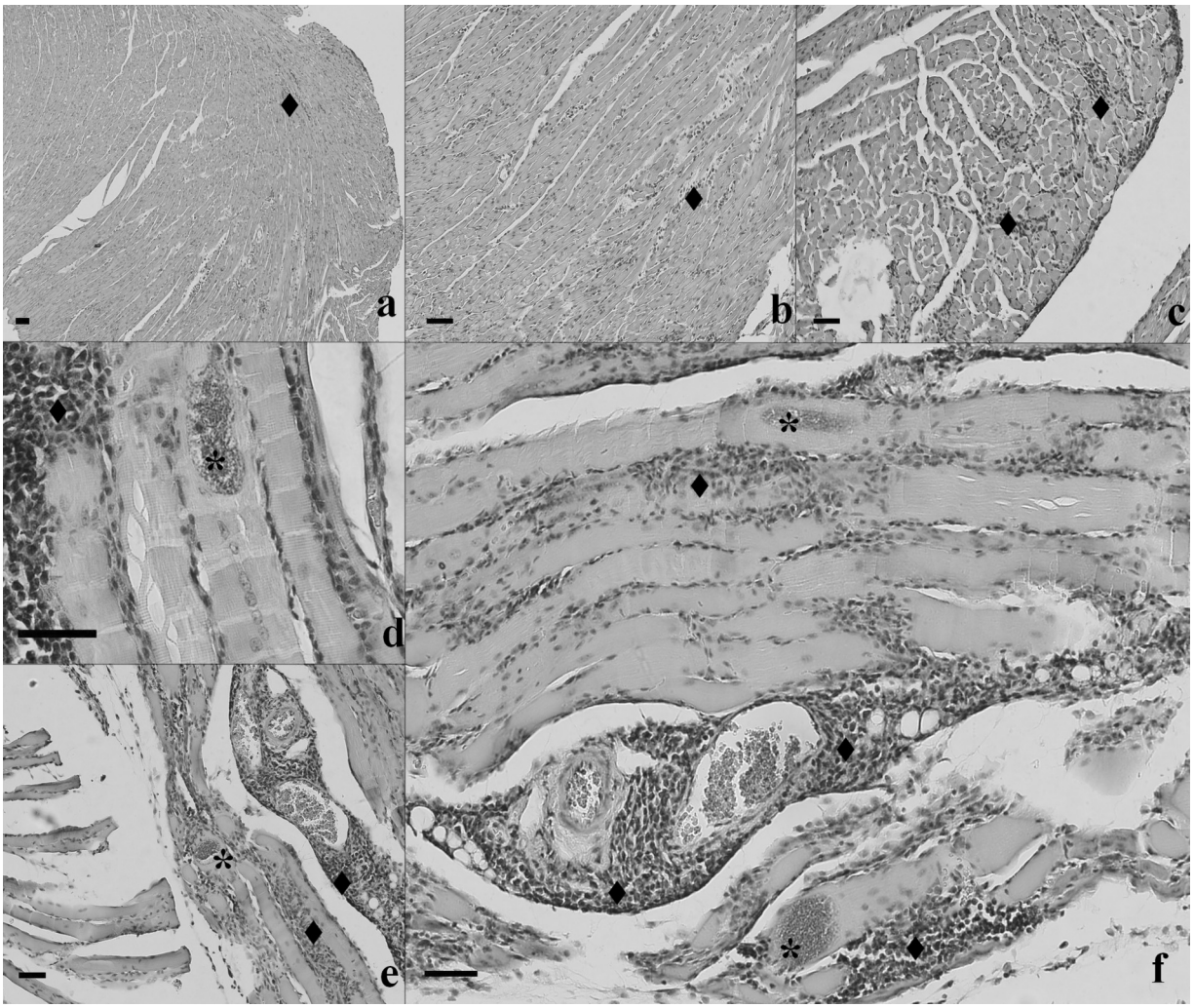

Figure 3. Myocardial (a, b, and c) and skeletal muscle (d, e, and f) sections from mice infected with Casibla, 35 days post infection (♦: inflammatory infiltrates; ⋆: amastigote nests; bar: 50 μm).

The inflammatory infiltrates in the cardiac samples from **group 3** were minor, covering an average area of 0.87%; however, amastigote nests were present in most of the samples analyzed (Figure 5 a, b, and c). The skeletal muscle samples presented mild to severe inflammatory infiltrates, covering an average area of 7.37% of the sections analyzed; several amastigote nests were also found in all the samples (Figure 5 d, e, and f).

No statistical differences were found between the groups when the areas covered by inflammatory infiltrates in cardiac and skeletal muscles were compared.

3.3. *T. cruzi* detection in host's tissues

T. cruzi specific PCRs were performed in order to verify the presence of the parasite in the different tissues from the infected individuals. As can be observed in Table 3, specific PCRs (TCZ) were positive for all the samples (blood, cardiac and skeletal muscles, liver, and spleen) from the single-infected mice (groups 1 and 2). Interestingly, 2/3 spleen samples from the double-infected mice (group 3) were negative for the presence of *T. cruzi*; the rest of the samples from the double-infected mice were positive.

3.4. *Molecular characterization of the parasites present in each tissue*

Due to the fact that the studies were performed using two parasite isolates (and not pure strains or stocks), the parasites from *each* tissue were characterized using two different typing methods for DTU determination: a set of three molecular markers (**mini-exon**, **24Sα rRNA,** and **18S rRNA**) as described in Figure 1 and a set of two molecular markers (**TcSC5D** amplicon digested with Hpa I and Sph I, and **TcMK** amplicon digested with Xho I) as described in Figure 2. Table 3 shows the results obtained after the molecular characterization using each method.

The isolate used to infect **group 1** (Casibla) consisted of a mixture of lineages II and VI (two samples from this group were not determined, but they are expected to correspond to lineage VI as some of the rest of the samples), being both lineages present in most of blood, cardiac and skeletal muscle samples; all liver and spleen samples however, only showed the presence of lineage VI. The isolate used to infect **group 2** (Lucky) also consisted of lineages II and VI; both lineages were present in most of the samples in this case. *T. cruzi* lineage distribution in **group 3** (double-infected) was similar to that found in group 2: most samples presented a mixture of lineages II and VI, although some samples only showed the presence of lineage VI.

Table 3
Detection and molecular characterization of the parasites present in blood, heart, skeletal muscle, liver, and spleen of *T. cruzi* infected mice during the acute phase of the experimental infection (results from 3 mice from each group are shown).

Group	Molecular Marker 100 bp	Mouse 1					Mouse 2					Mouse 3					Lineage controls	
		B	C	SM	L	S	B	C	SM	L	S	B	C	SM	L	S	Y strain	Tulahuen strain
Group 1	TCZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	β-actin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	ME	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300
	24S	125	130	125	125	125	125	125	125	ND	125	125	125	125	125	125	125	110
	18S	165	165	NA	NA	NA	165	165	165	NA	NA	165	NA	165	NA	NA	165	NC
	***T. cruzi* Lineage (*)**	**II**	**II**	**VI**	**VI**	**VI**	**II**	**II**	**II**	**V o VI?**	**VI**	**II**	**VI**	**II**	**VI**	**VI**	**II**	**V**
	TcSC5D + Hpa I and Sph I →																		
	TcMK + Xho I →						ND			ND					-			-	
	***T. cruzi* Lineage (**)**	**VI**	**VI**	**VI**	**VI**	**VI**	**V or VI?**	**VI**	**VI**	**V or VI?**	**VI**	**VI**	**VI**	**II**	**VI**	**VI**	**II**	**V**
Group 2	TCZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	β-actin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	ME	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300
	24S	125	125	125	125	125	125	125	125	125	125	125	125	125	125	125	125	110
	18S	165	165	165	165	165	165	165	165	165	NA	165	165	165	165	165	165	NC
	***T. cruzi* Lineage (*)**	**II**	**II**	**II**	**II**	**II**	**II**	**II**	**II**	**II**	**VI**	**II**	**II**	**II**	**II**	**II**	**II**	**V**
	TcSC5D + Hpa I and Sph I →		ND																
	TcMK + Xho I →	ND							-									-	
	***T. cruzi* Lineage (**)**	**V or VI?**	**II or VI?**	**VI**	**VI**	**VI**	**VI**	**VI**	**II**	**VI**	**VI**	**VI**	**VI**	**VI**	**VI**	**VI**	**II**	**V**
Group 3	TCZ	+	+	+	+	-	+	+	+	+	+	+	+	ND	+	-	+	+
	β-actin	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	-	-
	ME	300	300	300	300	-	300	300	300	300	300	300	300	ND	300	-	300	300
	24S	125	125	125	125	-	125	125	125	125	125	125	125	ND	125	-	125	110
	18S	165	165	165	165	-	165	165	165	165	165	NA	165	ND	NA	-	165	NC
	***T. cruzi* Lineage (*)**	**II**	**II**	**II**	**II**	**-**	**II**	**II**	**II**	**II**	**II**	**VI**	**II**	**ND**	**VI**	**-**	**II**	**V**
	TcSC5D + Hpa I and Sph I →					-									ND		-		
	TcMK + Xho I →				ND	-									ND		-	-	
	***T. cruzi* Lineage (**)**	**VI**	**VI**	**VI**	**V o VI?**	**-**	**VI**	**VI**	**VI**	**VI**	**VI**	**VI**	**VI**	**ND**	**VI**	**-**	**II**	**V**

B: blood; C: cardiac muscle; SM: skeletal muscle; L: liver; S: spleen. Numbers are given in base pairs. NA: no amplification; NC: non corresponding; ND: not described.
* *T. cruzi* lineage determination according to Ramírez *et al.* (2010) algorithm, using three molecular markers (mini-exon, 24Sα rRNA and 18S rRNA). *See section 2.5 and figure 1 for details.*
** *T. cruzi* lineage determination according to Cosentino and Agüero (2012), using two molecular markers (**TcSC5D** amplicon digested with Hpa I and Sph I and **TcMK** amplicon digested with Xho I). *See section 2.5 and figure 2 for details.*
Arrows in the 100bp ladders point to the 500 bp marker. The last two columns include the characterization of two known *T. cruzi* strains: Y strain (Tc II) and Tulahuen strain (Tc V), for comparison.

Table 4
Samples with different RFLP patterns in tissues to those found in blood, after the digestion of the mini-exon fragment with 5 different restriction enzymes.

	Samples with different RFLP patterns in tissues to those found in blood																			
	Bce AI				Dra III				Fsp I				Rsa I				Bsm AI			
	C	SM	L	S	C	SM	L	S	C	SM	L	S	C	SM	L	S	C	SM	L	S
Group 1	3/3	3/3	3/3	3/3	1/3	1/3	0/3	2/3	1/3	1/3	0/3	2/3	1/3	1/3	0/3	1/3	1/3	1/3	0/3	0/3
Group 2	1/3	1/3	2/3	3/3	1/3	1/3	0/3	1/3	1/3	1/3	0/3	0/3	1/3	1/3	0/3	0/3	1/3	1/3	0/3	0/3
P [1]	<0.0001	<0.0001	<0.0001	>0.99	>0.99	>0.99	>0.99	<0.0001	>0.99	>0.99	>0.99	<0.0001	>0.99	>0.99	>0.99	<0.0001	>0.99	>0.99	>0.99	>0.99
Group 3	1/3	2/2	2/3	1/1*	0/3	1/2	0/2	0/1*	0/3	2/3	1/3	0/1*	0/3	2/3	1/3	0/1*	0/3	1/1	0/3	0/1*

C: cardiac muscle; SM: skeletal muscle; L: liver; S: spleen.
[1] P: Group 1 vs. Group 2; Pearson Chi-square test.
* Only one spleen sample from group 3 was positive for *T. cruzi* presence.

3.5. RFLP studies

To further study *T. cruzi* variability in the host's tissues (blood, heart, skeletal muscle, liver, and spleen), RFLP studies were performed using the previously amplified mini-exon fragment. Table 4 shows the amount of samples with different patterns in tissues to those found in blood of the same animal, obtained after the digestion with 5 restriction enzymes (Bce AI, Dra III, Fsp I, Rsa I, and Bsm AI). Bce AI proved to be more efficient at differentiating the parasite populations present in each tissue.

Considering heart, skeletal muscle, liver, and spleen samples together from **group 1**, 100% of tissue samples (digested with Bce AI) showed different RFLP patterns to those found in circulation of the same host. Bearing in mind that, as stated in the previous section (Section 3.4), all blood samples from this group corresponded to lineages II and VI, and that lineage II was not present in 67% of the tissue samples, these results are expected. However, variations were also found even in the samples infected with the same lineages, suggesting different parasite populations within a given lineage.

As stated earlier, the rest of the enzymes were less efficient at differentiating parasite populations in each tissue, showing a lower percentage of different RFLP patterns between blood and tissues of the same individual: 33% of tissue samples digested with Dra III or Fsp I, 25% of tissue samples digested with Rsa I, and 16% of samples digested with Bsm AI. Noteworthy is the fact that the tissue samples with patterns similar to those found in circulation were identified as such either by Dra III or Fsp I.

A lower percentage of **Group 2** tissue samples were found to have different RFLP patterns to those found in the circulating parasites of the same host: 58% of the tissue samples digested with Bce AI; 25% of the samples digested with Dra III; and 17% of the samples digested with Fsp I, Rsa I, or Bsm AI. Considering that 83% of tissue samples were infected with the same lineages that were found in circulation (II and VI), these results highlight the greater homogeneity between circulating and tissue parasite populations, in contrast to what was found in group 1. However, some variations were still found in the samples infected with the same lineages.

Fragments obtained in tissue samples from **group 3** were, in general, less variable than those found in tissues from groups 1 and 2; the majority of the fragments were more similar to those found in group 1, suggesting supremacy of the parasite populations present in this isolate (Casibla). In this case, the percentages of tissue samples with different RFLP patterns to those found in circulation were: 67% for Bce AI, 30% for Fsp I or Rsa I, and 13% for Dra III or Bsm AI.

Worth mentioning is the fact that some samples presented bands comparable to those obtained for lineage II and for lineage VI combined, suggesting the presence of both lineages together in the same tissue, corroborating the results found in the previous section (Section 3.4).

4. Discussion

The biological behavior of the different *Trypanosoma cruzi* strains/isolates is still under research and the importance of exploring the relevance of these differences in natural isolates is of great significance. There has been substantial progress in understanding the biological and genetic diversity of the parasite, as well as the population polymorphisms associated with susceptibility to the disease. However, many other aspects, such as host-parasite interactions, genetic mechanisms of cellular interaction, genetic variability, and tropism, are not known enough (Ferreira Bellini et al., 2012).

The molecular epidemiology of *T. cruzi* may have important implications on the disease features. While no global correlations

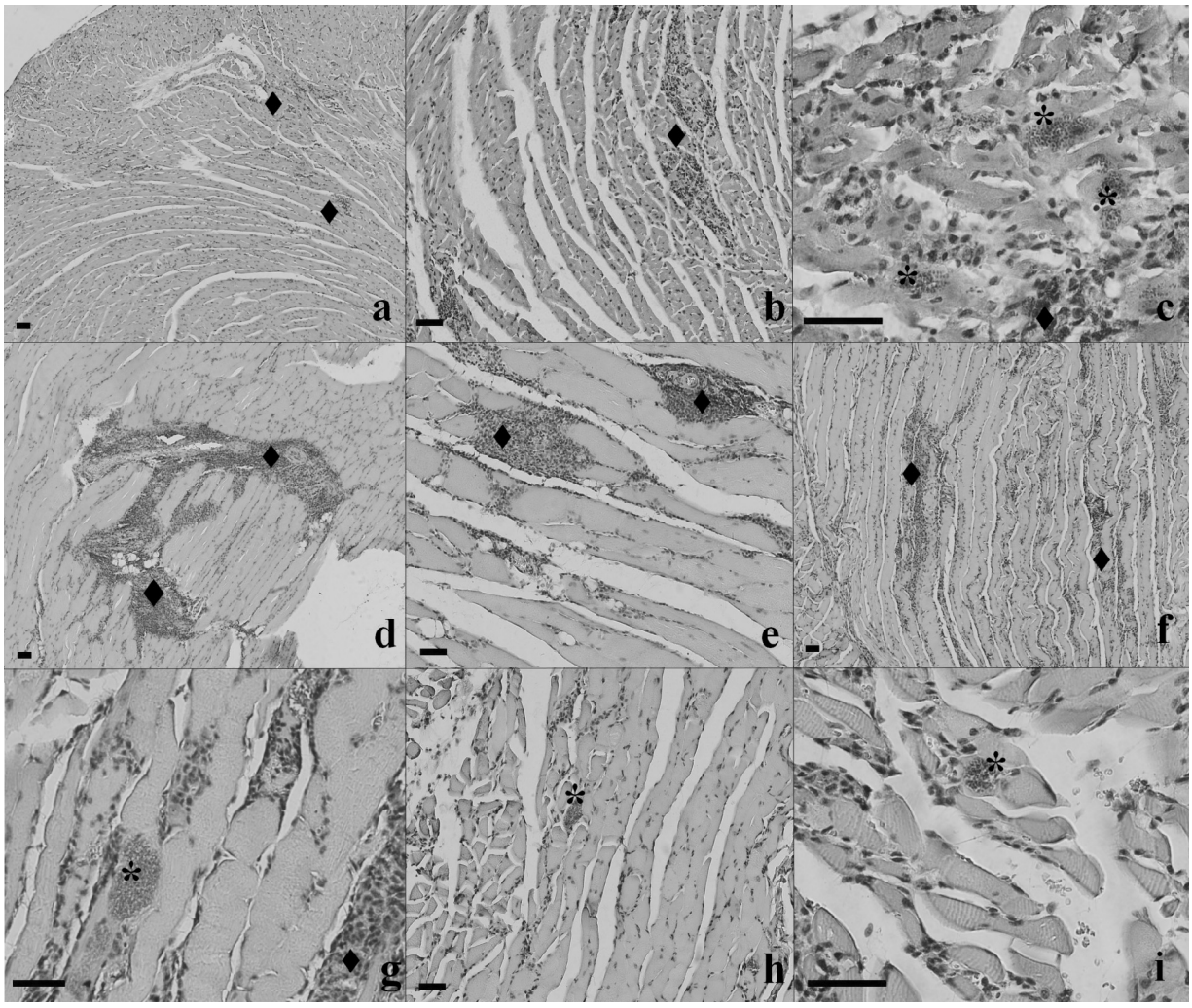

Figure 4. Myocardial (a, b, and c) and skeletal muscle (d–i) sections from mice infected with Lucky, 35 days pot infection (♦: inflammatory infiltrates; ⋆: amastigote nests; bar: 50 μm).

have been found relating *T. cruzi* genetic variability and the disease outcome (Mantilla et al., 2010), there are many instances of local correlations between parasite genetics/biology and the clinical form of the disease (Campbell et al., 2004; Guhl and Ramírez, 2013). The geographic variations in the prevalence of the different clinical forms of Chagas disease are likely due to both the genetic variation of *T. cruzi* and the genetic and environmental features of the host (Macedo and Pena, 1998; Macedo et al., 2004).

The importance of studies focused on the biological behavior of isolates in coinfections has been highlighted (Ragone et al., 2012), under the assumption that they could interact and modify the intrinsic behavior of individual isolates, inducing different biological properties, or altering the transmission dynamics. In the present work we evaluated the presence and distribution of two *T. cruzi* isolates (both obtained from the umbilical cord of newborn child) in blood, cardiac and skeletal muscles, liver, and spleen tissues from mice infected with either a single isolate or a combination of two of them, during the acute phase of the experimental infection. Both isolates used for the experimental infections consisted of a mixture of two different *T. cruzi* lineages (Tc II and Tc VI), as was determined by the molecular characterization studies. This is in accordance with the theoretical expectation, since patients in endemic areas are likely to be infected by multiple contacts with different triatomines and these, in turn, may feed on different infected individuals, resulting in a mixture of parasites within the same host (human, vector, or reservoir). Several studies had been conducted exploring the biological properties and behaviors of the different *T. cruzi* DTUs separately (Aquilino et al., 2012; Ragone et al., 2012; Silva Valadares et al., 2012); few however have focused on isolates consisting of a mixture of different DTUs (Llewellyn et al., 2011), such as the present.

The group infected with a combination of the two isolates together presented a peak in circulating parasites earlier and that lasted for a longer period of time than the groups infected with a single isolate. This was probably due to the fact that this group was initially inoculated with a higher amount of parasites than the single-infected groups (100 and 50 trypomastigote forms, respectively); the total amount of parasites in circulation however, was intermediate between the curves of each single-isolate infected group. On the other hand, this higher parasite initial inoculum did not impact on the survival since all groups (single and double-infected) had a survival rate close to 30% by day 35 postinfection.

As expected for the acute phase, the three groups presented positive specific PCRs for the blood samples. The parasite presence was also confirmed by the histological studies and specific PCR in tissues (heart, skeletal muscle, liver, and spleen) from animals from the different groups. While most of the cardiac and the skeletal muscle samples from the group infected with the two isolates together showed amastigote nests in the histological studies, the samples from the groups infected with a single isolate presented

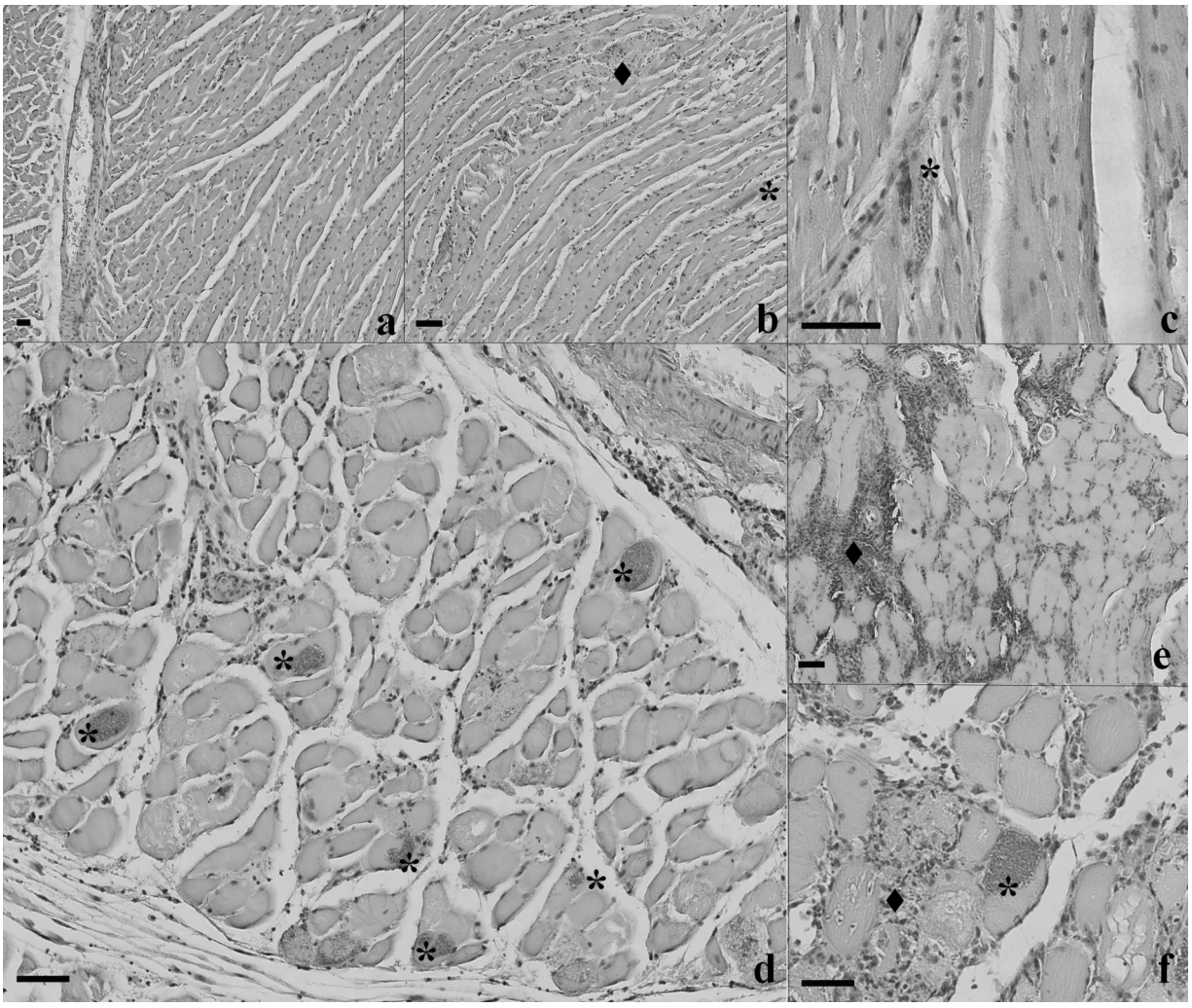

Figure 5. Myocardial (a, b, and c) and skeletal muscle (d, e, and f) sections from mice infected with Lucky + Casibla, 35 days pot infection (♦: inflammatory infiltrates; ⋆: amastigote nests; bar: 50 μm).

inflammatory infiltrates but few parasite nests. Again, these results could be related to the higher parasite initial inoculum, but also could be due to parasite/parasite interactions that modify the behavior of individual isolates (Ragone et al., 2012). Since the past decade, the application of PCR to detect *T. cruzi* directly in blood and tissue samples has opened new possibilities for the diagnosis of infection and evaluation of trypanocidal chemotherapy in different clinical and epidemiological settings (Duffy et al., 2009; Marcet et al., 2006; Moser et al., 1989). As expected using a more sensitive method for the detection of the parasite, even the samples with no amastigote nests turned out to be positive in the PCR studies.

In most cases, the skeletal muscle was the tissue with the highest number of amastigote nests, confirming the importance of these organs as a reservoir of parasites during the infection. Additionally, within each group, the area covered by inflammatory infiltrates found in the skeletal muscle was higher than the area covered by inflammation in the heart; similar results have been described for the cardiac chronic stage of infection (Lo Presti et al., 2009). No differences were found between groups regarding the amount of inflammatory infiltrates in the tissues analyzed. The presence of the parasite in the hosts' tissues from early moments of the infection (as confirmed by the presence of several amastigote nests in the tissues) would probably influence the outcome of the later infection and reaffirms the importance of specific treatment to eliminate it. Taking these results into consideration, a relevant approach would therefore be the etiological therapy using carrier molecules, that bind benznidazole or any other trypanocidal drug and takes it inside the tissues, improving both solubility and parasite specificity (Buckner and Navabi, 2010; Silva et al., 2008).

Several studies have confirmed the high incidence of multiple infections in humans and vectors (Bosseno et al., 2000; Mantilla et al., 2010; Solari et al., 2001), which highlights the relevance of present results since the group infected with both isolates together presented several amastigote nests in the tissues (both in cardiac and skeletal muscle), significantly higher than those present in the single-isolate infected groups, even when each isolate also consisted of a mixture of parasites. The relevance of this double-infected group lies in the fact that the distribution of any particulate type of parasite could be modified by the presence of other types of parasites. Andrade and collaborators performed experiments with double-infected models, also demonstrating that different *T. cruzi* clones presented distinct tissue distributions: while one strain predominated in the heart and did not infect the rectal tissue, the other was preponderant in the rectum and all other tissues, with exception of the heart (Andrade et al., 1999; 2002). Mixed infections with parasites corresponding to different DTUs in the same patient have been reported (Mantilla et al., 2010; Ramírez et al., 2010); the isolates used in the present study (which were obtained from newborn child from endemic area), are examples of these mixed infections and their use in an experimental model of infection has

shown that the different "types" of parasites could prefer certain organs or tissues and spread throughout the host, which would worsen the scenario for treatment strategies. These results agree with the "Clonal-Histotropic Model" of Chagas disease proposed by Macedo (2002) that describes how natural strains of *T. cruzi* become multiclonal and how this could affect disease pathogenesis.

Present results show that parasite populations present in certain organs (heart, skeletal muscle, liver, or spleen) would differ from the ones present in blood, as was observed for all the individuals infected with isolate 1 (Casibla). Additionally to the presence of different DTUs or lineages in the same isolate with differential tissue tropism, high variability within a given lineage and even within a given tissue was also found through the RFLP studies, showing diverse parasite populations in the different tissues even when infected with one particular lineage. Parasites in bloodstream and cardiac tissue have also been previously found to be different (Andrade et al., 2002; Macedo et al., 2004; Manoel-Caetano et al., 2007). These results, highlight the necessity to conduct studies based on the use of tissue and bloodstream samples from infected patients to compare the *T. cruzi* genotypes present in the same individual (Ramírez et al., 2010; Cura and Schijman, 2013), since they may be quite different. An important matter to address here is the fact that using the method with three molecular markers (figure 1), lineage assignments are, in some cases, based on the absence rather than the presence of specific PCR products (Zingales et al., 2012), which can be problematic since the presence of certain lineages (particularly lineages III, IV, and VI) could be covered by the presence of other lineages (V and II, respectively) in the same tissue. For this reason, we used a different method for parasite classification, based on two additional molecular markers (figure 2), to identify the parasite lineage present in each tissue. This alternative method has recently been proposed for the discrimination of major *T. cruzi* lineages by the amplification of two products followed by a RFLP analysis (Cosentino and Agüero, 2012). As a consequence of this later method, most samples that were classified as lineage II with the first method (particularly from group 2) showed that lineage VI was also present in those tissues. In fact, this alternative method only showed the presence of lineage VI in these samples, and lineage II presence was covered in this case; this is a consequence of both lineages sharing the resulting restriction fragments (see figure 2). The presence of both lineages in some tissues was also supported by the RFLP analysis (with Bce AI, Fsp I, Rsa I, Dra III, and Bsm AI), since these samples presented a combination of bands that corresponded both to lineage II and lineage VI samples. Due to the nature of the methods used for the molecular characterization, it seems important to use both of them together to achieve a more precise determination of the type of parasite present in each tissue, since the presence of certain lineages can be covered by the presence of others in each case. Several authors are searching for new and alternative characterization methods to overcome these difficulties (reviewed in Zingales et al., 2012; Cura and Schijman, 2013).

Another important issue to address is the heterogeneity of the results obtained from the animals within a given group and the lack of correlation between a particular parasite population (differentiated by the RFLP studies) and the tissue tropism; this suggests that any particular *T. cruzi* population could infect different tissues and their presence in any particular organ would be determined by complex host/parasite interactions. Further studies are needed to clarify these interactions and the importance of tissue tropism in the development of the infection. It is important to note that host immune response also modulates the parasite tissue tropism: it has been demonstrated that the host immune system can vary the number of tissue-bound as well as the number of circulating parasites that are available for infection (Andrade et al., 2010). Different immunological components have been implicated in host modulation of *T. cruzi* infection: major histocompatibility complex (MHC) locus (Tarleton et al., 1996), NF-κβ production (Hall et al., 2000), and cytokine profiles (Bahia-Oliveira et al., 1998; Powell et al., 1998) among others. Outbred mice, such as the ones used in the present work, have individually different immunological features, including unique MHC haplotypes, a locus that has been associated with the tissue distribution pattern of *T. cruzi* infection (Freitas et al., 2009): heterogeneous results regarding parasite tissue distribution were also found in double-infected models using outbred Swiss mice, while the same pattern was found in animals of a more similar genetic background (inbred BALB/c, DBA-2, and C57BL/6 mice) (Andrade et al., 2002). Moreover, the patterns of parasite tissue distribution were similar for BALB/c and DBA-2 mice which share the same MHC haplotype (H-2^d), but different for C57BL/6 mice which have a different MHC haplotype (H-2^b), underlining the influence of the host MHC gene region in the differential tissue distribution of *T. cruzi* strains in these mice (Freitas et al., 2009).

Regardless the heterogeneity of the results, also highly due to the low number of mice used in each case, consequent of the high mortality, the use of natural isolates for experimental infection models (as opposed to cloned parasite cells) allows a more representative scenario of the infection, closer to what actually takes place in natural infections.

Several authors have demonstrated the different susceptibility/resistance of *T. cruzi* strains or isolates to standard treatments (Caldas et al., 2008; Filardi and Brener, 1987; Martins et al., 2007; Toledo et al., 2003), which would be further accentuated if these differences are present in one infected individual (blood vs. tissues), as found in the present study, or even within a particular tissue of the same individual. Knowledge about complexity of *T. cruzi* strains/isolates is essential for determining the aspects involved in differential parasite tissue tropism, clinical manifestations of the disease, and drug resistance (Silva Valadares et al., 2012; Cura and Schijman, 2013). Present results will contribute to the knowledge of the complex interactions between *T. cruzi* and the host and will be useful to determine the prognosis of mixed infections, characteristic of endemic areas, and stimulate the search for novel treatment strategies taking into consideration the high variability of the parasite within a single host.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank Grupo Pilar-GEPSA for donating the pelleted food for the animals. This work was supported by grants from the Secretaría de Ciencia Técnica (SECyT) from Universidad Nacional de Córdoba and from Universidad Nacional de La Rioja, FONCYT (PICT-2007-01620), Ministerio de Ciencia y Tecnología de la Provincia de Córdoba and CONICET.

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