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TcI, TcII and TcVI *Trypanosoma cruzi* samples from Chagas disease patients with distinct clinical forms and critical analysis of *in vitro* and *in vivo* behavior, response to treatment and infection evolution in murine model

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ABSTRACT

The clonal evolution of *Trypanosoma cruzi* sustains scientifically the hypothesis of association between parasite's genetic, biological behavior and possibly the clinical aspects of Chagas disease in patients from whom they were isolated. This study intended to characterize a range of biological properties of TcI, TcII and TcVI *T. cruzi* samples in order to verify the existence of these associations. Several biological features were evaluated, including *in vitro* epimastigote-growth, "Vero"cells infectivity and growth, along with *in vivo* studies of parasitemia, polymorphism of trypomastigotes, cardiac inflammation, fibrosis and response to treatment by nifurtimox during the acute and chronic murine infection. The global results showed that the *in vitro* essays (acellular and cellular cultures) TcII parasites showed higher values for all parameters (growth and infectivity) than TcVI, followed by TcI. *In vivo* TcII parasites were more virulent and originated from patients with severe disease. Two TcII isolates from patients with severe pathology were virulent in mice, while the isolate from a patient with the indeterminate form of the disease caused mild infection. The only TcVI sample, which displayed low values in all parameters evaluated, was also originated of an indeterminate case of Chagas disease. Response to nifurtimox was not associated to parasite genetic and biology, as well as to clinical aspects of human disease. Although few number of *T. cruzi* samples have been analyzed, a discreet correlation between parasite genetics, biological behavior *in vitro* and *in vivo* (murine model) and the clinical form of human disease from whom the samples were isolated was verified.

Abbreviations: DTU, discrete typing unit; TcI, *Trypanosoma cruzi* DTU I; TcII, *Trypanosoma cruzi* DTU II; TcIII, *Trypanosoma cruzi* DTU III; TcI,V, *Trypanosoma cruzi* DTU IV; TcV, *Trypanosoma cruzi* DTU V; TcVI, *Trypanosoma cruzi* DTU VI; MG, state of Minas Gerais, Brazil; PPP, pre-patent period; PP, patent period; MPP, maximum peak of parasitaemia; DMPP, day of the maximum peak of parasitemia; MOR, mortality; LIT, liver infusion tryptose; PBS, phophate buffer solution; rpm, rotation per minute; FBE, blood test fresh; PCR, polymerase chain reaction; Hm, hemoculture; d. a. t, day after treatment; FC-ALTA, research of live anti-trypomastigotes antibody; bp, base pair; MW, molecular weight; ITAP, group infected treated acute phase; ITCP, group infected treated chronic phase; NF, Nifurtimox.

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1. Introduction

Chagas disease or American trypanosomiasis, caused by the hemoflagellate protozoan *Trypanosoma cruzi,* was discovered in 1909 by Chagas (1909) and are present in twenty one countries in the Americas (WHO, 2015). New cases of Chagas disease are daily diagnosed in several countries and continents, originally considered non-endemic for Chagas disease (WHO, 2015)where the disease has being transmitted independent of the natural vectors of the parasite such as blood transfusion, congenital, transplantation of organs and tissues and others (Schmunis and Yadon, 2010).

The short-term acute phase of Chagas disease evolves to distinct clinical manifestations during long-lasting chronic phase, ranging from asymptomatic to cardiac, digestive (megacolon and/or megaesophagus) or cardiac/digestive clinical forms (Rassi et al., 2010).

Currently, six distinct *T. cruzi* genetic subdivisions or DTU's (discreet typing units) have been identified (Zingales et al., 2009). The clonal nature of *T. cruzi* reproduction and propagation is expected to be the basis of genetically stable populations, although the phenomenon of hybridization may rarely occur in this specie (Tibayrenc and Ayala, 2015). For these reasons, *T. cruzi* infection is considered the best model for to study the population genetics and the molecular epidemiology. However, the correlation between the genetic variability and the presence of different clinical forms is still questionable (Lages-Silva et al., 2006; D'Ávila et al., 2009). Several studies have intended to correlate the high rate of *T. cruzi* genetic variability to its biological characteristics (Toledo et al., 2002) as well as clinical manifestations of Chagas disease, but still unsuccessfully (Macedo et al., 2004).

Different hypotheses have been proposed in an attempt to elucidate the failure to establish this correlation. One of them may be resultant of the mixtures of different *T. cruzi* subpopulations of distinct genotypes that may lead to different infection evolution and clinical pictures (Macedo and Pena, 2002). Another hypothesis relates the fact that the disease is the result of a multifactorial process that involves aspects of the host, but also of the parasite. The inadequate choice of genetic targets may result in lack of correlation between the clinical forms of Chagas disease with the *T. cruzi* genetic variability. Moreover, the occurrence of mixed infection, with distinct *T. cruzi* populations, and the isolation of the predominant *T. cruzi* genotype in peripheral blood may also appear as a confounding factor in the attempt of the detection of association between parasite genetic and the clinical forms of Chagas disease (Macedo et al., 2004).

Taking into account the current *T. cruzi* division into distinct genetic groups and that the biological parameters of the parasite strains influence the infection evolution and probably the clinical aspects of Chagas disease (Macedo et al., 2004), we believe that to better understand the current disease of an endemic region firstly is necessary to know the parasite genetic profile affecting the local population. Therefore, the knowledge of parasite genetics would offers notions about patient's treatment decision, the clinical aspects of the disease, as well as how to establish better epidemiological surveillance and control strategies for Chagas disease (Coura and Junqueira, 2015; Martinez-Perez et al., 2016).

Considering these important statements, the current study aims to characterize a range of biological features of TcI, TcII and TcVI *T. cruzi* samples from Chagas disease patients with distinct clinical forms of the disease in order to verify possible correlations among the biological aspects of the parasites and the clinical aspects of the patients from whom these samples were isolated.

2. Methods

2.1. *T. cruzi* samples

The *T. cruzi* samples were obtained from chronic Chagas disease patients of the endemic areas, Berilo and Januária, Minas Gerais (MG) State, Brazil with different clinical forms. The six *T. cruzi* samples were properly genotyped by us (Oliveira et al., 2015) as previously proposed by Lewis et al. (2009) and D'Ávila et al. (2009). The criteria of D'Ávila et al. (2009) genotyping was adopted in order to identify safely the distinct DTUs as well as mixed infections in the samples. No evidences of mixed infection were verified by Oliveira et al. (2015). Samples of TcII and TcVI DTUs were chosen because they are present in the endemic region where we have worked in the last years. Although TcI samples were not detected in Berilo, one sample of this DTU was included due its high distribution and importance in Chagas disease epidemiology.

The only sample identified as TcI genotype (sample code: Pr 150) was isolated from a patient presenting the cardiac clinical form (Abolis et al., 2011); three samples were identified as TcII genotype (sample code: 501, 452, 728) and were isolated from patients presenting the indeterminate, cardiac and cardiac/digestive clinical forms, respectively; and two samples were identified as TcVI genotype (sample code: 1337 and 748) and were isolated from patients presenting the indeterminate and cardiac/digestive clinical forms, respectively. The patients were clinically classified according to Brazilian Consensus in Chagas disease (Ministério da Saúde, 2005), based on anamnesis, clinical and physical examination, thoracic and contrasted gastrointestinal tract X-ray along with electrocardiogram after consensus between two independent physicians. The inclusion of patients in the study occurred after signature of the Informed Consent approved by the Ethics Committee on Human Research at the Centro de Pesquisa René Rachou, FIOCRUZ, Belo Horizonte, MG, Protocol: 007/2002 and at the Universidade Estadual de Maringá, Paraná state, Brazil. The Table 1 summarizes the main features of *T. cruzi* samples evaluated.

All *T. cruzi* samples were isolated by hemoculture (HC) technique as previously described by Chiari et al. (1989). Parasites were growth in LIT medium and stored in liquid nitrogen at first passage in this medium for further use in this study.

2.2. T. cruzi *growth in acellular culture* in vitro

T. cruzi growth *in vitro* was evaluated for each sample in LIT medium culture plus 10% of fetal bovine serum starting with a standard inoculum of 1.0×10^7 parasites/mL in a final volume 3 mL (Nogueira-Paiva et al., 2015). The cultures were maintained in biological incubator BOD (FANEM® model 347) at 28 °C ± 1 °C. The number of epimastigotes was monitored daily, for 20 consecutive days, in Newbauer chamber examined in optical microscopy (4X40). All experimental batches were carried out in triplicate and data reported as number of epimastigotes/mL.

2.3. T. cruzi *growth in "Vero" cells culture* in vitro

The *T. cruzi* amastigote growth in "Vero" cells were evaluated as proposed by Andrade et al. (2010) modified as follows: The cells previously stored in liquid nitrogen stocks were after three passages in D-EMEN seeded into 75 cm^2 polystyrene sterile bottles containing 14 mL of D-MEM medium (Dulbecco's Modified Eagle Medium, Invitrogen, Carlsbad, CA, USA), supplemented with 5% of heat-inactivated fetal bovine serum (FBS, Nutricell, Campinas, SP, Brazil), added of 2.5% 1 M HEPES pH 7.2, 1.0% of 2 mM glutamine/ml, 0.1% mercaptoetanol 50 mM/mL, 0.2% of gentamicin sulfate 200 mg/mL (Schering-Plough, Kenlworth, NJ, USA). Semiconfluent "Vero" cells monolayer were reached approximately 72 h

Table 1
Main features of *T. cruzi* isolates.

T. cruzi Genotype	Sample code	Host-source Features			
		Gender	Age (years)	Clinical Form	City/State
*Tc*I	PR150	Mas	57	Cardiac	Januária (MG)
TcII	501	Fem	22	Indeterminate	Berilo (MG)
*Tc*II	452	Fem	61	Cardiac	Berilo (MG)
*Tc*II	728	Mas	72	Cardiac/Digestive	Berilo (MG)
*Tc*VI	1337	Fem	52	Indeterminate	Berilo (MG)
*Tc*VI	748	Fem	58	Cardiac/Digestive	Berilo (MG)

after seeding. The "Vero" cells were then trypsinized and approximately $4{,}0 \times 10^4$ cells were transferred to each well of glass cover slips and incubated at 5% CO^2 and at 37 °C overnight, prior infection (10:1 ratio) with metacyclic *T. cruzi* trypomastigotes previously differentiated in Grace's medium as described by Contreras et al. (1985). Twenty four hours after infection, the "Vero" cells monolayers were washed with phosphate buffer-saline (PBS) to remove extracellular parasites and the cultures maintained under the same conditions during 24, 48 and 72 h. Following, the "Vero" cells cultures were fixed in methanol and stained with Fast Panotic dye, and amastigotes growth examined under optical microscopy (100× magnification). The "Vero" cells infectivity was defined for each *T. cruzi* sample in triplicates and the results expressed as: mean percentage of infected cells per 100 cells; the number of amastigotes/cell and the number of amastigotes per total number of infected cells.

2.4. In vivo *studies in murine model*

For this study female Swiss mouse, 18 and 20 g, 30 days old were used. The parameters parasitemia, bloodstream polymorphism, mortality and response to etiological treatment were evaluated in mice because they are associated to each other (Andrade, 1974) and consequently linked to the parasite genetics (Andrade and Magalhães, 1996; Toledo et al., 2002).

For each sample of *T. cruzi* four groups of eight mice (total 192) were inoculated intraperitoneally with 10,000 blood trypomastigotes/animal quantified according to Brener (1962), previously maintained up to three passages in Swiss mice. Two groups for each sample of *T. cruzi* (16 mice) were used for the biological characterization and one was the control untreated group; two others (16 mice) were used for treatment of the acute phase – AP (treatment started in the first day of patent parasitemia) and chronic phase – CP (treatment started on the 90th day of infection) with nitrofuran (nifurtimox, NF) Lampit® produced by Bayer Health Care. Parasitemia was assessed daily (starting at day 4 up to day 50 after infection) by fresh blood examination (FBE) according to Brener (1962). The biological parameters from parasitemia curves were evaluated for each *T. cruzi* sample, including:

Pre-patent period (PPP): corresponding to the period (days) between *T. cruzi* inoculation and the day before the first parasite detection by FBE. The PPP results were expressed as mean ± standard deviation;

Patent Period (PP): corresponding to the time span between the first and the last day of positive FBE. The PP results were expressed as mean ± standard deviation;

Maximum Peak of parasitaemia (MPP): corresponding to the maximum number of trypomastigotes/0.1 mL of blood detected by FBE. The MPP results were expressed as mean ± standard deviation;

Day of Maximum Peak of Parasitemia (DMPP): corresponding to the day of maximum of parasitemia occurrence. The DMPP results were expressed as mean ± standard deviation;

Morphological characterization of bloodstream trypomastigotes: correspond to the relative percentage of thin, intermediary and large bloodstream trypomastigotes forms observed under optical microscopy (100X) according to Brener and Chiari (1963) methodology.

Mortality (MOR): The mortality of the animals was assessed daily until the 90th d.a.i. and weekly until 360th d.a.i. The results were expressed in cumulative percentages.

All *in vivo* studies in murine models were carried out at Centro de Ciência Animal (CCA-UFOP), according to the guidelines established by the Conselho Nacional de Controle em Experimentação Animal (CONCEA) and following the international guidelines. The study was previously approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Ouro Preto (UFOP) under the protocol number 26/2014.

2.5. *Response to nifurtimox treatment during acute and chronic murine infection*

2.5.1. *Therapeutic schemes*

The nifurtimox treatment was carried out by oral route using gavage during the acute phase (starting at the first day of patent parasitemia) and chronic phase of infection (performed at 90 d.a.i.), using daily dose of 100 mg/Kg of body weight for 20 consecutive days, according to Brener (1962).

The analysis of the infection evolution and therapeutic efficacy was performed considering several parameters. As cure control was adopted the negativation of three distinct categories of tests: parasitological methods (HC and polymerase chain reaction – PCR) and conventional serological test (IgG anti-*T. cruzi* enzyme-linked immunosorbent assay – ELISA), as suggested by Ministério da Saúde (2005), and non-conventional serology (flow cytometric anti-live trypomastigotes antibodies – IgG antibody by FC- ALTA), as proposed by Martins-Filho et al. (1995) modified, performed at days 90, 180 and 360 after treatment, as briefly described below:

2.5.2. *Hemoculture (HC)*

The HC was performed as described by Filardi and Brener (1987).

2.5.3. *Polymerase chain reaction (PCR)*

The PCR was performed as previously described by Gomes et al. (1998) modified, using the primer #121 (AAATAATGTACGGGTGAGATGCATGA) and primer #122 (GGTTCGATTGGGGTTGGTGTAATATA). The amplified DNA was visualized by electronic gel electrophoresis (QIAXCEL Advanced Qiagen). Positive and negative control samples and reagents controls were always examined in parallel.

2.5.4. *Conventional serology (ELISA)*

The ELISA to detect IgG anti-*T. cruzi* antibodies was performed according to Voller et al. (1975) modified by Santos da et al. (2012).

2.5.5. *Nonconventional serology (FC-ALTA)*

The FC-ALTA to detect IgG anti-live trypomastigote antibodies was performed as originally described by Martins-Filho et al. (1995) modified for murine model by Molina et al. (2000).

Based on the cure criteria used, the *T. cruzi* samples were further classified according to the therapeutic response as resistant (cure rates ≤ 33%), partially susceptible (cure rates >33% < 67%) and susceptible (cure rates ≥ 67%), according to Toledo et al. (2003).

2.6. Cardiac histopathological analysis

Cardiac histopathological analyzes were performed during acute and chronic phases, before and 360 days after nifurtimox treatment. The cardiac inflammatory infiltrate and fibrosis were evaluated in hematoxylin-eosin and Trichrome of Masson respectively, in stained 5 μm tissue sections analyzed in optical microscopy (40X). The inflammation analysis was performed using a Leica QWin software (Leica Microsystems, Wetzlar, Germany). Data were expressed as the mean number ± standard deviation of nucleated cells in a total area of 0.8×10^6 μm^2, as previously described by Maltos et al. (2004). Collagen deposition was carried out by qualitative comparative analysis in tissues stained with Trichrome Masson.

2.7. Statistical analysis

Overall comparative analysis of epimastigote growth curve profiles from distinct *T. cruzi* genotypes was performed considering the area under the curve (AUC) *via* Kruskall-Wallis followed by Dunn's post-test. Significant differences at $p \leq 0.05$ were identified by letters "a", "b", "c", "d", "e" and "f" as compared to TcI (Pr 150), TcVI (1337), TcII (501), TcII (452), TcII (728) and TcVI (748), respectively. Additionally, comparison amongst distinct *T. cruzi* genotypes at specific days were carried out by Kruskall-Wallis followed by Dunn's post-test. AUC values are provided in the figures. Day of maximum parasite growth is identified by arrows.

The *in vitro* "Vero" cells infectivity obtained for distinct *T. cruzi* genotypes was evaluated by the two-way analysis of variance ANOVA for comparison of the number of infected cells, number of amastigotes and the ratio of amastigotes/infected cells. Significant differences at $p \leq 0.05$ amongst distinct *T. cruzi* genotypes were identified by letters "a", "b", "c", "d", "e" and "f" as compared to TcI (Pr 150), TcVI (748), TcII (501), TcII (452), TcII (728) and TcVI (1337), respectively. Moreover, differences amongst the incubation periods (24, 48 and 78 h) were identified by connecting lines.

Comparative analysis of parasitemia curves observed for distinct *T. cruzi* genotypes was performed considering the area under the curve *via* ANOVA, followed by Newman-Keuls post-test. AUC values are provided in the figures. Comparison amongst distinct *T. cruzi* genotypes at specific days were carried out by Kruskall-Wallis followed by Dunn's post-test. Day of maximum parasite growth is identified by arrows. Analysis of biological parameters derived from the parasitemia curve (PPP, PP, MPP and DMPP) was performed by ANOVA followed by Newman-Keuls post-test. In all cases, significant differences at $p \leq 0.05$ were identified by letters "a", "b", "c" and "d" as compared to TcII (501), TcII (452), TcII (728) and TcVI (1337), respectively. Observational analysis of relative percentage of bloodstream trypomastigote forms was also performed.

Comparative analysis of parasitemia curves obtained for distinct *T. cruzi* genotypes, before and after nifurtimox treatment at acute phase, was performed considering the area under the curve *via* ANOVA followed by Newman-Keuls post-test. AUC values are provided in the figures. Significant differences at $p \leq 0.05$ were identified by connecting lines.

The comparison of cardiac inflammatory infiltrated observed for distinct *T. cruzi* genotypes was carried out by Kruskall-Wallis followed by Dunn's post-test. Significant differences at $p \leq 0.05$ were identified by letters "a", "b", "c" and "d" as compared to TcII (501), TcII (452), TcII (728), and TcVI (1337), respectively. Additionally, comparisons of cardiac inflammatory infiltrated before and after nifurtimox treatment during acute and chronic phase was carried out by Man-Whitney and significant differences at $p \leq 0.05$ were identified by connecting lines.

All statistical analysis and graphical arts were performed using the GraphPad Prism software (version 5.03, San Diego, CA, USA).

3. Results

3.1. In vitro *epimastigote growth curve of TcI, TcII and TcVI* T. cruzi *samples in acellular culture*

The overall epimastigote growth curve profiles for TcI, TcII and TcVI *T. cruzi* samples in LIT medium are shown in Fig. 1. Data analysis demonstrated that TcII 728 sample presented higher AUC ($p \leq 0.05$) as compared with TcI (Pr 150) sample. No differences were observed amongst the other *T. cruzi* samples. Additional analysis demonstrated that TcI (Pr 150) presented a late maximum peak of parasite growth around 16th day in culture. Both TcVI samples presented maximum peak of parasite growth around 8th day in culture. Interestingly, TcII samples displayed distinct patterns of parasite growth with early maximum peak observed for 501 sample around 6th day, intermediate maximum peak observed for 452 around 8th day and a maximum peak observed for 728 around 10th day in culture (Fig. 1).

3.2. *Infectivity of TcI,TcII and TcVI* T. cruzi *samples in "Vero" cells culture*

The infectivity for "Vero" cells evaluated at 24, 48 and 72 h after infection for each *T. cruzi* sample is presented in Fig. 2. The final infectivity was similar for all samples of the three DTUs although one sample of TcII (501) and one of TcVI (1337) have grown more rapidly. The results showed that the 1337 sample (TcVI) led to an overall higher number of infected cells at 24 and 48 h after infection as compared to other *T. cruzi* samples. The TcII (501) sample yield higher number of amastigotes per cell at 72 h after infection in relation to all samples. Conversely, the TcII (501) sample also presented the highest ratio of amastigotes per infected cell at 72 h after infection (Fig. 2).

3.3. *Parasitemia curves in Swiss mice infected with TcI, TcII and TcVI* T. cruzi *samples*

The overall pattern of parasitemia observed during experimental murine infection with TcI, TcII and TcVI *T. cruzi* samples is shown in Fig. 3. Data demonstrated that although the TcI (Pr150) and TcVI (748) samples have infected mice, they did not presented patent parasitemia sufficient for inoculation of animals necessary for the comparative analysis with the other samples.

The TcVI (1337) sample induced low levels of parasitemia, evident by the low AUC (Fig. 3) and the lowest MPP values (Table 2). The TcII samples yield the highest parasitemia levels as observed by elevated AUC (Fig. 3) and highest MPP values (Table 2). Amongst the TcII sample, the sample 501 led to the most prominent parasitemia level, characterized by outstanding AUC and MPP values (Fig. 3 and Table 2).

The analysis of additional biological features derived from the parasitemia curve demonstrated that TcVI (1337) and TcII (728) samples presented similar and higher PPP as compared to TcII (501) and TcII (452) samples that showed similar PPP. The PP of TcII (501) and TcII (728) sample presented similar and higher PP values than TcVI (1337) and TcII (452) that showed similar PP. Analysis of DMPP demonstrated that the TcII (728) showed the latest peak of parasitemia observed around 27th day after infection as compared to all other samples evaluated (Table 2).

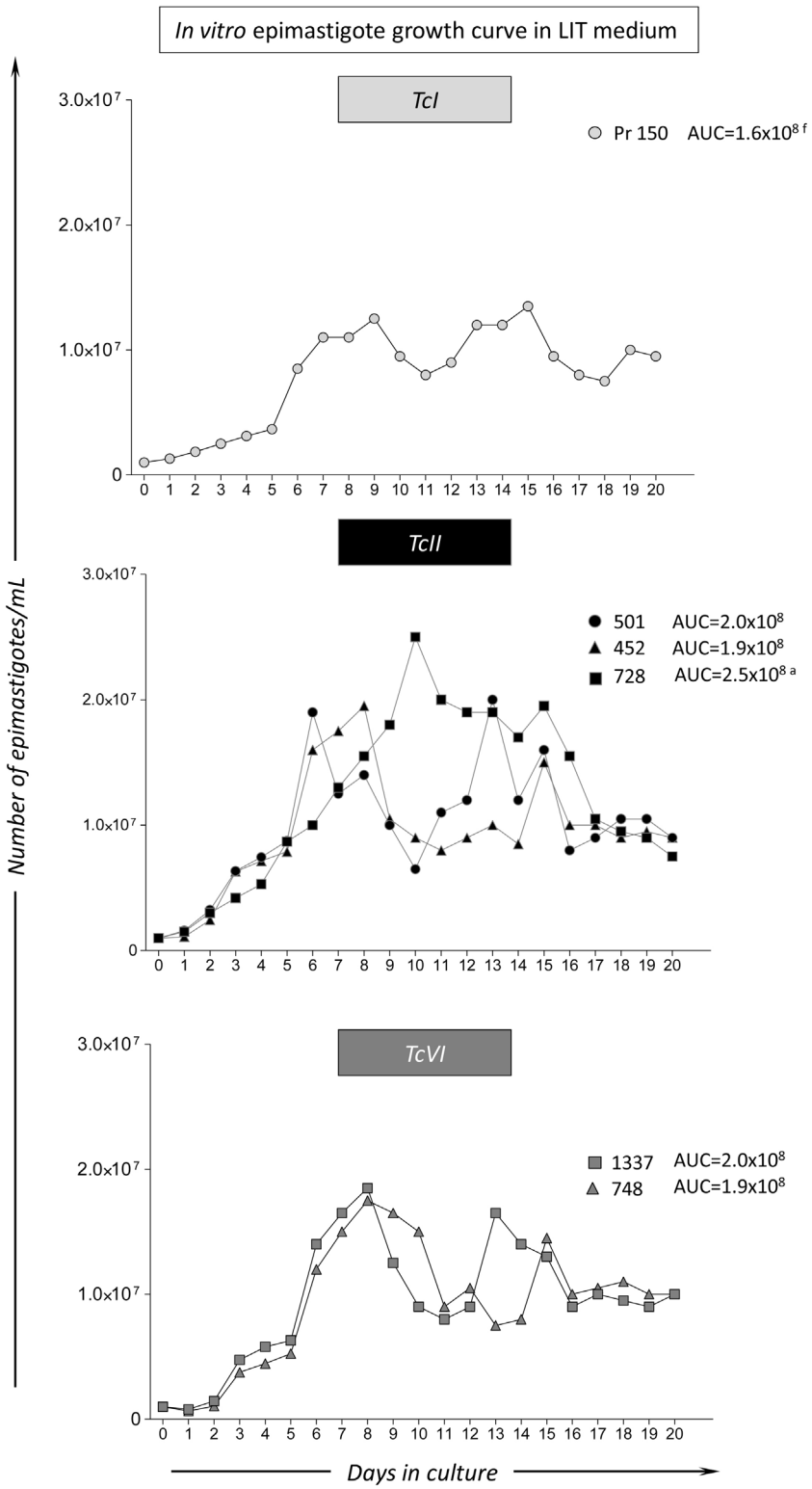

Fig. 1. *In vitro* epimastigote growth curve of TcI, TcVI and TcII *Trypanosoma cruzi* samples in acellular culture. All experimental batches were carried out in triplicate and data reported as number of epimastigotes/mL. Overall comparative analysis of epimastigote growth curve profiles from distinct *T. cruzi* genotypes was performed considering the area under the curve (AUC) *via* Kruskall-Wallis followed by Dunn's post-test. Significant differences at $p \leq 0.05$ were identified by letters "a", "b", "c", "d", "e" and "f" as compared to TcI (Pr 150 =), TcVI (1337 = 1337), TcVI (748 =), TcII (501 = ●), TcII (452 = ▲) and TcII (728 = ■), respectively. AUC values are provided in the figures. Day of maximum peak of parasite growth is identified by arrows.

3.4. Morphological analysis of bloodstream trypomastigotes in Swiss mice infected with TcII and TcVI T. cruzi *samples*

The analysis of TcII sample revealed that despite the difference in the length of patent period, all TcII samples displayed a similar kinetic of changes in morphological features of bloodstream trypomastigotes during the evaluation. Indeed, all TcII samples displayed an early predominance of intermediate trypomastigotes that were subsequently changed towards large trypomastigote forms.

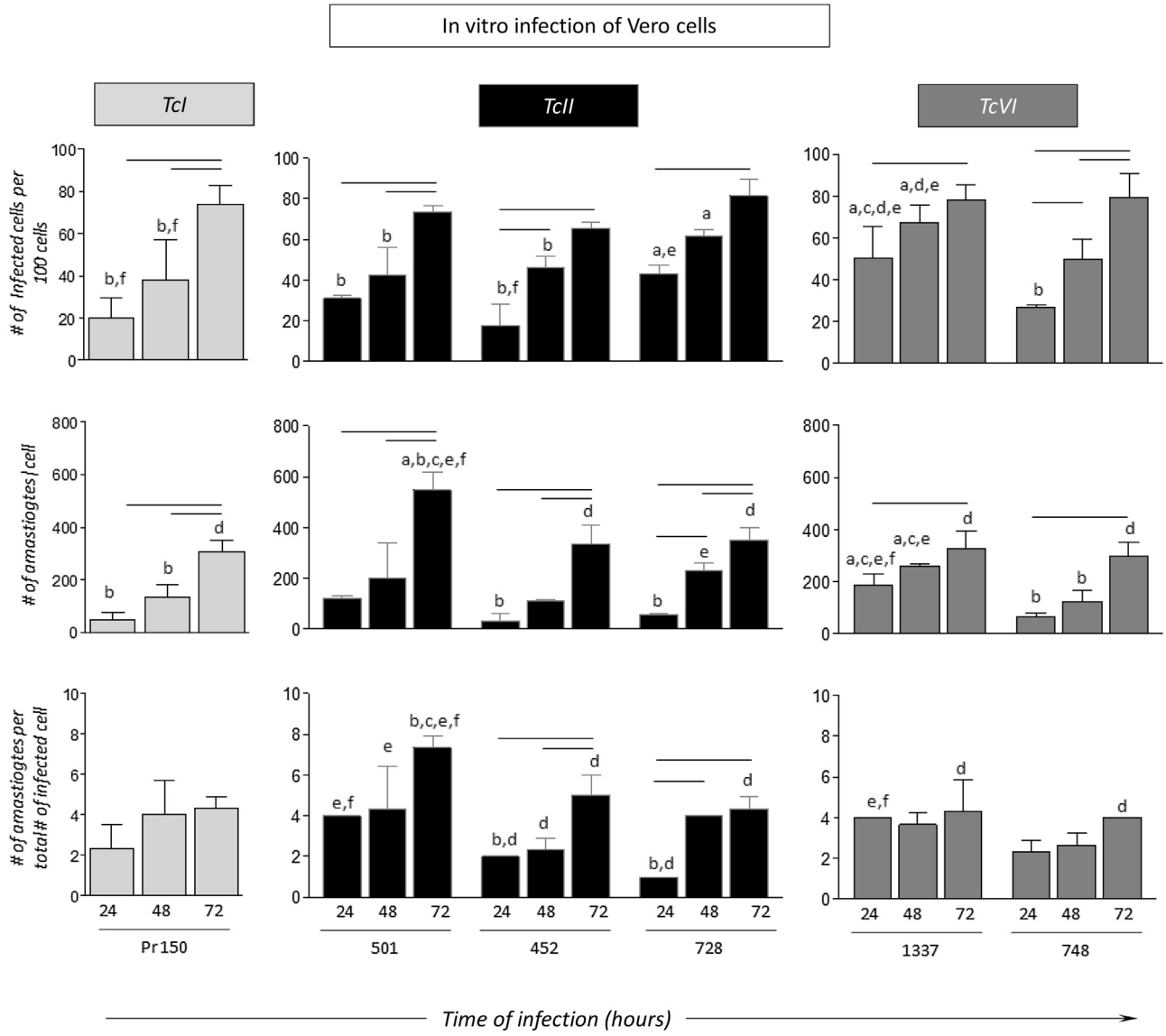

Fig. 2. Infectivity of TcI (▭), TcVI (▬) and TcII (■) *Trypanosoma cruzi* samples in Vero cells culture *in vitro*. The Vero cells infectivity was defined at 24, 48 and 72 h after infection for each *T. cruzi* sample in triplicated and the results expressed as mean percentage of infected cells per 100 cells; the number of amastigotes/cell as well as number of amastigotes per total number of infected cells. Significant differences at $p \leq 0.05$ amongst distinct *T. cruzi* genotypes were identified by letters "a", "b", "c", "d", "e" and "f" as compared to TcI (Pr 150), TcVI (1337), TcVI (748), TcII (501), TcII (452) and TcII (728), respectively. Moreover, differences amongst the incubation periods (24, 48 and 78 h) were identified by connecting lines.

Table 2
Biological parameters from parasitemia curves of *T. cruzi* isolates in murine model.[a]

T. cruzi Genotype (Isolate code)	Biological parameters			
	PPP	PP	MPP	DMPP
TcII [501]	$6.8 \pm 0.8^{a,d}$	$21.3 \pm 3.3^{a,c}$	$200.0 \pm 40.5^{a,c,d}$	17.5 ± 1.4
TcII [452]	$6.9 \pm 0.3^{a,d}$	$13.0 \pm 1.9^{b,d}$	$102.5 \pm 44.9^{a,b}$	13.3 ± 1.3
TcII [728]	$12.3 \pm 0.5^{b,c}$	$28.9 \pm 2.1^{a,c}$	$100.0 \pm 49.1^{a,b}$	26.9 ± 1.3
TcVI [1337]	$10.3 \pm 0.9^{b,c}$	$16.1 \pm 2.4^{b,d}$	$48.5 \pm 16.5^{b,c,d}$	23.5 ± 1.4

[a] Data are expressed as mean ± standard deviation for all parameters (n = 8). PPP: pre-patent period; PP: patent period; MPP: maximum peak of parasitemia; DMPP: Day of maximum peak of parasitemia.

Data analysis revealed that the infection with TcVI (1337) sample displayed an early predominance of thin forms (approximately 71%) that was subsequently replaced by a floating proportion of intermediate and large forms, with the latter being predominant late during the evaluation (Fig. 4).

3.5. *Response to nifurtimox treatment during acute and chronic murine infection with TcII and TcVI* T. cruzi *samples*

Data regarding the parasitemia during the acute infection and acute/chronic cardiac inflammatory infiltrates observed during experimental infection with TcII and TcVI *T. cruzi* samples are provided in Fig. 5. The results demonstrated that regardless the

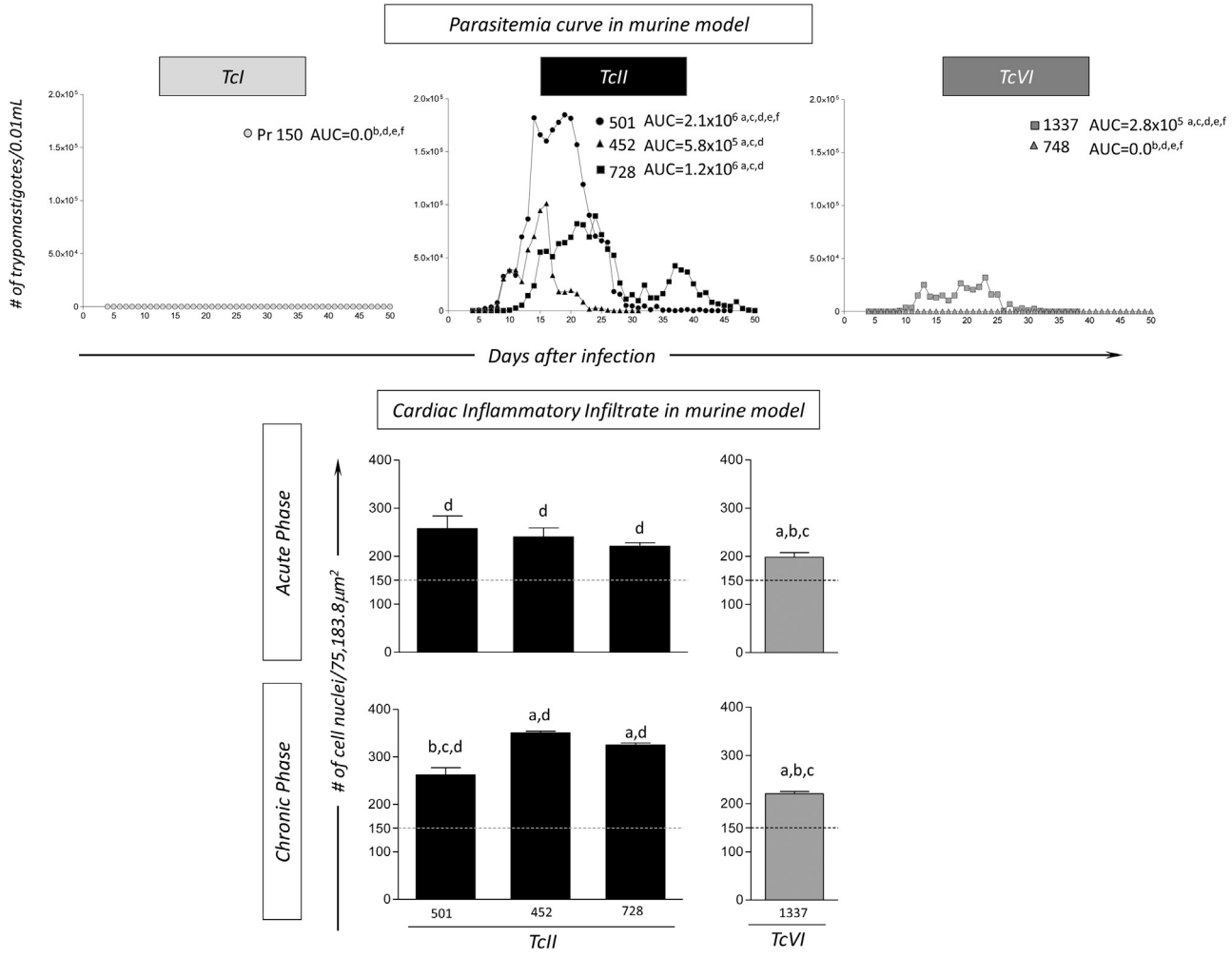

Fig. 3. Parasitemia curves and cardiac inflammatory infiltrate analysis in Swiss mice infected with TcI, TcII and TcVI *Trypanosoma cruzi* samples. Parasitemia data are reported as number of trypomastigotes per 0.01 mL of blood animals infected with TcI (Pr 150 = ○), TcVI (1337 =), TcVI (748 =), TcII (501 = ●), TcII (452 = ▲) and TcII (728 = ■). Comparative analysis of parasitemia curves observed for distinct *T. cruzi* genotypes was performed considering the area under the curve *via* ANOVA followed by Newman-Keuls post-test. AUC values are provided in the figures. The cardiac inflammatory infiltrate was evaluated in animals infected with TcVI ( ) and TcII ( ) *Trypanosoma cruzi* samples. Data are reported as the mean number ± standard deviation of nucleated cells in a total area of 0.8×10^6 μm^2. The comparison of cardiac inflammatory infiltrated observed for distinct *T. cruzi* genotypes was carried out by Kruskall-Wallis followed by Dunn's post-test. In all cases, significant differences at $p \leq 0.05$ were identified by letters "a", "b", "c" and "d" as compared to TcVI (1337), TcII (501), TcII (452) and TcII (728), respectively.

parasitemia levels, the nifurtimox treatment during acute infection was able to reduce the parasitemia to almost undetectable levels in animals infected with TcII and TcVI *T. cruzi* samples (Fig. 5).

3.6. *Effectiveness of nifurtimox treatment during acute and chronic murine infection with TcII and TcVI* T. cruzi *samples*

The nifurtimox therapeutic effectiveness was assessed using two parasitological methods (HC and PCR) along with conventional (ELISA) and non-conventional serology (FC-ALTA) performed at day 90, 180 and 360 after treatment (Table 3).

The therapeutic response was evaluated according to the cure criteria assessment adapted from that originally proposed by Ministério da Saúde (2005) and Krettli et al. (1982), using the PCR as an additional parasitological method and the ELISA and FC-ALTA methods as conventional and non-conventional serological approaches, respectively. Adopting the classic criterion of cure proposed by the Ministério da Saúde (2005), only two animals infected with *T. cruzi* sample 501(TcII) and treated in the acute phase were considered cured. However, using the criteria proposed by Krettli et al. (1982) modified, it was demonstrated that nifurtimox treatment during acute infection led to higher cure rates in mice infected with TcII (501) and TcII (452) samples (75 and 88% of cure respectively). Lower cure rate was observed in animals infected with TcII (728) sample of this same genotype that displayed 25% of cure. Animals infected with TcVI (1337) were partially resistant (63% of cure) to nirfurtimox treatment. An overall therapeutic failure was observed during chronic infection for both criteria of cure (Table 3).

3.7. *Cardiac inflammatory infiltrate analysis in Swiss mice infected with TcI, TcII and TcVI* T. cruzi *samples*

Analysis of cardiac inflammatory infiltrate during experimental murine infection with TcII and TcVI *T. cruzi* samples demonstrated that the TcVI (1337) sample led to significant lower cardiac inflammation at both acute and chronic infection. Interestingly, regardless the similar cardiac inflammation triggered by all TcII samples at acute infection, the TcII (501) induced lower cardiac inflammation at chronic infection as compared to both TcII (452) and TcII (728) samples (Fig. 3).

Moreover, the nifurtimox treatment performed at acute infection was able to significantly reduce the cardiac inflammatory infiltrate in all infected animals, regardless the *T. cruzi* genotype and sample, the results became similar to the control group INT

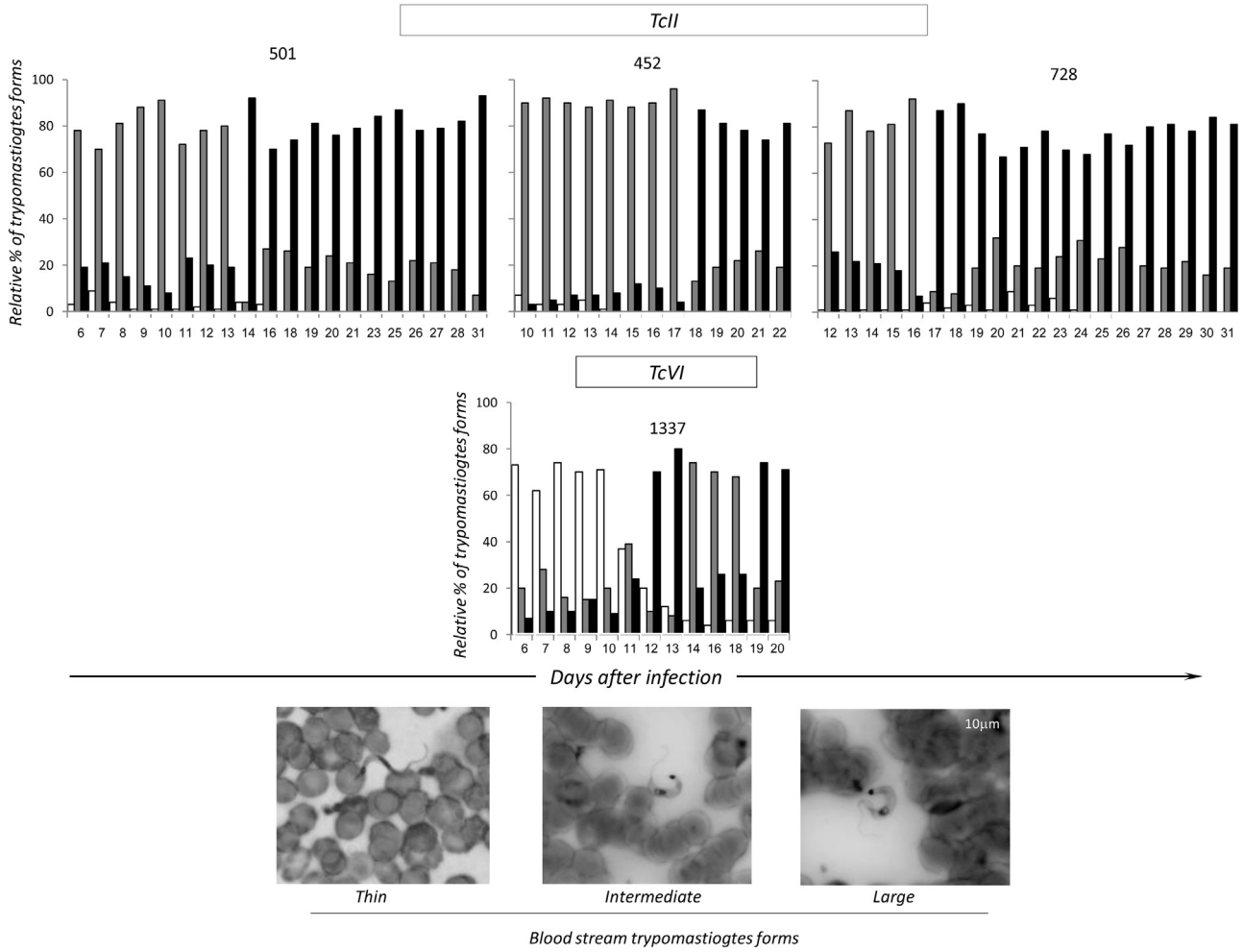

Fig. 4. Polymorphism of *Trypanosoma cruzi* bloodstream trypomastigotes in Swiss mice infected with TcII and TcVI *Trypanosoma cruzi* samples. Observational analysis of relative percentage of thin (▭), intermediary (▭) and large (■) bloodstream trypomastigotes forms observed under optical microscopy (100X). The results were expressed as relative frequency of each *T. cruzi* bloodstream trypomastigote form along the patent period. Representative images of each bloodstream trypomastigote form (thin, intermediary and large) are provided in the figure.

(Fig. 5). On the other hand, the nifurtimox treatment applied during chronic infection did not induce significant changes in the cardiac inflammation (Fig. 5).

3.8. *Fibrosis analysis in Swiss mice infected with TcI, TcII and TcVI* T. cruzi *samples*

Although apparently, has been verified discreet neoformation of collagen in the control groups (INT) than the treated ones, the quantitative analysis revealed similar collagen deposition in the heart of the animals; regardless the treatment had been done in the acute or chronic phases of infection (data not shown).

3.9. *Mortality of Swiss mice infected with TcI, TcII and TcVI* T. cruzi *samples*

No animal death was observed throughout the experimental acute and chronic phases of the infection up to day 360 after inoculation (data not shown).

4. Discussion

The *T. cruzi* species is divided into six distinct genetic lineages, better known as DTUs I, II, III, IV, V and VI (Zingales et al., 2009), that are distributed differently according to geographic region (Zingales et al., 2012). After established that the *T. cruzi* presents structure and clonal evolution (Tibayrenc et al., 1986), the correlation between the genetic and biological properties of the parasite (Buscaglia and Di Noia, 2003) is scientifically sustained and corroborated in several studies with strains and clonal stocks of the parasite *in vitro* (Laurent et al., 1997; Revollo et al., 1998), mice (Carneiro et al., 1991; Andrade and Magalhães, 1996; Toledo et al., 2002) and vectors (Lana et al., 1998). Therefore, the association of these features with the clinical forms of the disease in patients from whom the parasites were isolated is theoretically expected although some studies have failure in corroborate this hypothesis (Lages-Silva et al., 2006; D'Ávila et al., 2009).

Thus, the purpose of the present study was to characterize the biological features of *T. cruzi* samples (TcI, TcII and TcVI), isolated from chronic Chagas disease patients with distinct clinical forms, considering several parameters, including "*in vitro*" and "*in vivo*" biological properties along with the therapeutic response in acute and chronic murine infection, with the perspective of to detect some correlation of these parameters with the clinical characteristics of the disease in the patients from whom the parasites were obtained.

The growth of all six samples studied in LIT medium demonstrated that sample 728 of TcII genetic group presented the highest AUC when compared with TcI (Pr 150) and the other two samples of TcII DTU, which also showed fast or similar growth in LIT

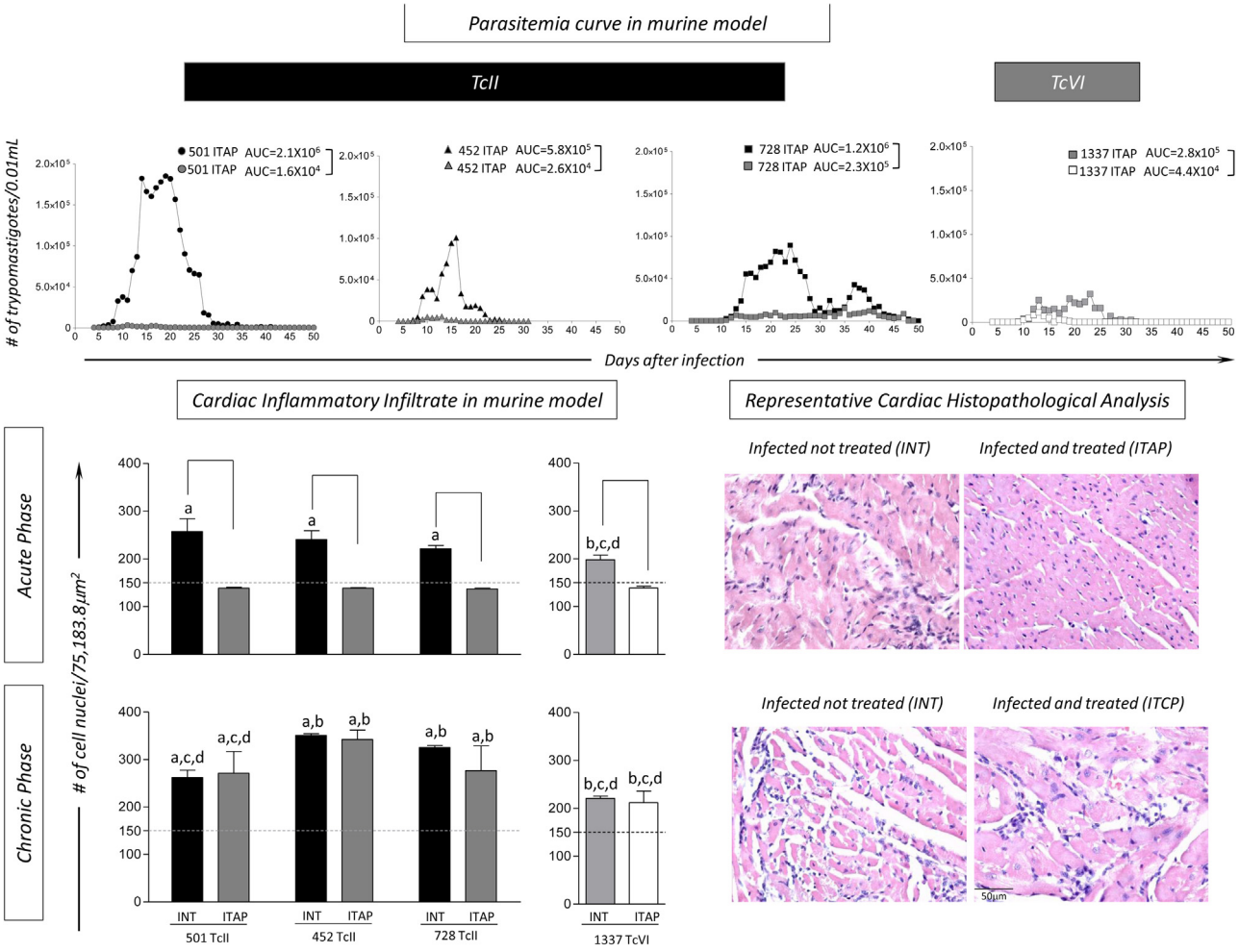

Fig. 5. Response to nifurtimox treatment during acute and chronic murine infection with TcII and TcVI *Trypanosoma cruzi* samples. Parasitemia data during acute phase are reported as number of trypomastigotes per 0.01 mL of blood of animal infected with TcVI (1337 = , □), TcII (501 = ●, ○), TcII (452 = ▲, ■) and TcII (728 = ■, ▲). Comparative analysis of parasitemia curves observed for distinct *T. cruzi* genotypes before and after nifurtimox treatment was performed considering the area under the curve *via* ANOVA followed by Newman-Keuls post-test. AUC values are provided in the figures. Significant differences at $p \leq 0.05$ were identified by connecting lines. The cardiac inflammatory infiltrate was evaluated in animals infected with TcVI (, □), and TcII (,) *Trypanosoma cruzi* samples. Data are reported as the mean number ± standard deviation of nucleated cells in a total area of 0.8×10^6 μm^2. The comparison of cardiac inflammatory infiltrated observed for distinct *T. cruzi* genotypes before and after nifurtimox treatment was carried out by Kruskall-Wallis followed by Dunn's post-test. Significant differences at $p \leq 0.05$ were identified by letters "a", "b", "c" and "d" as compared to TcVI (1337), TcII (501), TcII (452) and TcII (728), respectively. Additionally, significant differences at $p \leq 0.05$ for analysis carried out before and after nifurtimox treatment are highlighted by connecting lines. Representative images of changes observed after nifurtimox treatment applied at acute infection and the lack of impact of treatment during chonic infection are provided in the figure.

than the observed in TcVI sample. TcI (Pr 150) also presented a later maximum peak of growth in culture when compared with all other samples evaluated. Association between parasite genetic and behavior in LIT medium was also verified by Laurent et al. (1997) with clonal stocks of *T. cruzi*, Rimoldi et al. (2012) when evaluated *T. cruzi* samples isolated of domestic cats and vectors, and Nogueira-Paiva et al. (2015).

In "Vero" cells it was verified that the final infectivity of these cells at 72 h after culture was similar for all samples of all DTUs. However, the replication as amastigotes were fast in one sample of TcII (501) and one sample of TcVI (1337) in the previous evaluation (24, 48 h). The TcII (501) sample yield higher number of amastigotes per cell and the highest ratio of amastigotes per infected cell at 72 h after infection. On the other hand, TcI sample displayed the lowest growth in "Vero" cells. These results corroborate the study of Andrade et al. (2010) that also observed higher capacity of TcII genotype strains in infecting "Vero" cells than TcI strains for cardiomyocytes.

Several authors have demonstrated that *T. cruzi* samples from human cases of Chagas disease of different endemic areas usually have low virulence in mice, sometimes with parasitemia in sub-microscopic levels detected only by hemoculture, as shown by several publications (Abolis et al., 2011; Oliveira-Silva et al., 2015; Teston et al., 2013). Similarly, Sales-Campos et al. (2015) recently demonstrated that strains belonging to genotype TcI have subpatent parasitemia in mice. Rare exceptions exist such as VL-10 strain (TcII), also isolated from a human case of Virgem da Lapa, MG, (Filardi and Brener, 1987) very close to Berilo municipality, and considered highly virulent leading to intense inflammatory process associated to severe cardiac injury in dog models (Caldas et al., 2013). The TcI (Pr 150) sample here evaluated presented low subpatent parasitemia as previously demonstrated by Teston et al. (2013) and therefore was not further evaluated in mice model. Regarding TcII samples, Andrade et al. (2011) also demonstrated that all TcII samples, displayed parasitemia profile, virulence, mortality rate, histopathological lesions and tissue tropism consistent with previous reports for this *T. cruzi* genotype. In the present study, TcII and TcVI samples showed significant differences in biological parameters derived from the parasitemia curve, including PPP, PP, MPP, DMPP. In the present study, the majority of the samples stud-

Table 3
Efectiveness of nifurtimox treatment during acute and chronic murine infection with distinct *T. cruzi* isolates[a].

T. cruzi Genotype (Isolate Code)		Cure Assessement										
		Parasitological Methods (% of negative results)						Serological Methods (% of negative results)				Kretlli & Brener Cure Criteria
								Conventional			Non-conventional	
		HC			PCR			ELISA			FC-ALTA	
		90dat	180dat	360dat	90dat	180dat	360dat	90dat	180dat	360dat	360dat	360dat
Acute Phase	TcII (501)	63 (5/8)	13 (1/8)	100 (8/8)	13 (1/8)	100 (8/8)	100 (8/8)	0 (0/8)	25 (2/8)	25 (2/8)	75 (6/8)	75[d] (6/8)
	TcII (452)	100 (8/8)	100 (8/8)	100 (8/8)	25 (2/8)	100 (8/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	88 (7/8)	88[d] (7/8)
	TcII (728)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	63 (5/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	25 (2/8)	25[a,b,c] (2/8)
	TcVI (1337)	100 (8/8)	100 (0/8)	100 (8/8)	100 (8/8)	75 (6/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	63 (5/8)	63[d] (5/8)
Chronic Phase	TcII (501)	25 (2/8)	100 (8/8)	100 (8/8)	50 (4/8)	50 (4/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/32)
	TcII (452)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/32)
	TcII (728)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	50 (4/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/32)
	TcVI (1337)	50 (4/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/32)

[a] Data are presented as percentage of negative results in parasitological e aserological methods (number of negative aninals/total number of animals); dat: day after treatment HC: hemoculture; PCR: polymerase reaction chain; ELISA: IgG anti-*T. cruzi* epimastigote enzine-linked immunosorbent assay; FC-ALTA: Flow cytometric anti-live trypomastigote IgG antibody. Krettli & Brener cure criteria as proposed by Krettli & Brener (1982).

ied presented a relative low or subpatent parasitemia (PR-150 of TcI and 748 of TcVI) and when inoculated in mice the animals survived until the end of the experiments (one year of infection) without death consequent of the infection as observed in highly virulent strains. The sample 1337 (TcVI) showed significant differences in all parameters in relation to TcII samples, as observed by Andrade et al. (2011).

The absence of mortality in mice observed with all samples studied are consistent with other previous studies which have also revealed *T. cruzi* strains of low virulence for samples isolated from human infected of the Central and Southern regions of Brazil (Devera et al., 2002; Rimoldi et al., 2012; Oliveira-Silva et al., 2015). The polymorphism of the blood trypomastigotes of TcII samples is compatible with the expected for parasites of this genetic group (Type II or Zimodeme II) as demonstrated by Andrade (1974) and Andrade and Magalhães (1996) as well as for TcVI sample (hybrid or Zimodeme III).

Data regarding the therapeutic response to nifurtimox during acute infection demonstrated significant reduction in parasitemia of all *T. cruzi* samples evaluated revealed by the area under the curve of parasitemia (lower AUC and the maximum peak of parasitaemia – PMP). The FBE showed limitation to detect parasites in treated and not cured animals due its low sensitivity (Brener 1962; Teston et al., 2013). On the other hand, when hemocultures was performed in animals ITAP with negative FBE the parasite was detected in different percentages of the animals (0–100%) according to the genetic group and sample. When it was analyzed the post-treatment evaluation in animals ITCP, 69% (22/32) of the treated mice were negative in hemoculture. For this reason the use of hemoculture alone is not recommended in post-treatment evaluations due its low sensitivity mainly in certain genetic groups of *T. cruzi* involved in the infection (Toledo et al., 2002; Toledo et al., 2003).

The therapeutic effectiveness at acute and chronic infections was also assessed by molecular parasitological method. The PCR detected 41% (13/32) of treatment failure in animals ITAP and in 25% (8/32) of the animals ITCP. It is important to highlight the progressive negativation of the parasitological tests used in the successive evaluations (especially at 90 and 360 d.a.t.), differently than the observed with the conventional serology (ELISA).

The positivity of the conventional serology (ELISA) during acute and chronic infection was higher than the observed in parasitological methods (94%). Considering the classic cure criterion (WHO, 2002) these results are indicative of global treatment resistance of the samples to nifurtimox in mice (6.25% of cure). Only 25% of mice infected with the strain TcII (501) treated during acute infection presented negative ELISA at 180 d.a.t. Such discrepancies between parasitological (HC, PCR) and serological methods have also been described in mouse model (Miyamoto et al., 2008; Oliveira-Silva et al., 2015). Therefore, other evaluations of these animals are necessary in order to ascertain the seronegativation, similar to that observed in humans considered cured (Alessio et al., 2014; Lana and Martins-Filho, 2015). However, a decrease of the absorbance was observed throughout the successive evaluations.

In order to better investigate the results of alternative methods applied to post-treatment evaluation we used FC-ALTA in parallel to previous tests (HC, PCR, ELISA) 360 d.a.t., never described in the literature in mice model. We prolonged the time of evaluation because in a recent review about post-treatment evaluation and cure criteria in Chagas disease (Lana and Martins-Filho, 2015) we addressed the need of serological tests able to present early seronegativation. One of the tests applied with this purpose is the FC-ALTA serology that detects the anti- live-tripomastigotes antibodies indicative of active infection in the host. Several publications using this methodology displayed shortly negative results after specific treatment, both in humans (Wendling et al. (2011), and in experimental animals (Martins et al., 2007; Oliveira-Silva et al., 2015). In this work, globally 64% of the animals treated during acute infection evaluated 360 d.a.t. were negative for FC-ALTA. Moreover, it was noteworthy that the parasitological tests (HC and PCR) of the animals were always negative, and in these mice no inflammation and collagen deposit in heart tissue were observed. It was verified higher cure rate by FC-ALTA, with negative results ranging from

25% to 88% when compared with the ELISA (0% of cure). Besides such results of FC-ALTA, the absorbance of ELISA in the animals was decreasing in the successive evaluations, as happened with HC e PCR positivity. although we have evaluated drug response in only one sample of tcvi, it was observed similarity of the rates of cure between TcII (501, 452 and 728) and TcVI (1337) samples.

The apparent lack of correlation between parasite genetic and the response to treatment of mice infected with TcII and TcIV samples here evaluated can be understood since TcVI parasites are resultant from hybridization phenomena between TcI and TcII (Westenberger et al., 2006) and the only sample of this genetic group here studied presented biological behavior more similar to TcII, which is also more genetically closer to TcII than TcI. Similar results were observed regarding treatment for clonal stocks of *T. cruzi* in hybrid group (Toledo et al., 2003) where parasites resistant, partially resistant and resistant to benznidazole were observed. Unfortunately the other sample of this DTU (TcVI) was not evaluated regarding treatment due its low parasitemia insufficient for inoculation of a group of mice.

When FC-ALTA were used in the evaluation of animals ITCP the results were 0% negative as well as ELISA, indicative of therapeutic failure of NF in the chronic phase of infection taking at consideration the classic and novel cure criteria. Thus, in this phase of infection no correlation between response to treatment and parasite genetic was observed. Probably and again, the low number of samples evaluated was not sufficient to verify this correlation, differently from the observed by Toledo et al. (2003), which working with 20 clonal stocks of *T. cruzi* verified total resistance or partial resistance in parasites of the genetic group I (TcI), partial resistance in the hybrid groups and higher susceptibility of genetic group II (TcII) to benznidazole. However, it is important to note that again, the parasitological tests (HC and PCR) carried using the peripheral blood of the animals showed tendency to negativation (100%), in parallel to decrease of absorbance of the serological tests ELISA (data not shown).

This great discrepancy in the rates of cure using the novel cure criterion for animal ITAP and ITCP may be interpreted as similar as the classically considered for Chagas disease infections in humans (Viotti et al., 2011; Machado-de-Assis et al., 2012) and experimental models (Guedes et al., 2004; Oliveira-Silva et al., 2015). The facility for cure the acute phase of the infection and the difficulty for cure the chronic phase of the infection considering the negative conventional serology as cure criterion. Other possible interpretation of these results is the long duration of the antibodies of the conventional serology (ELISA) following treatment in relation to antibodies of the non-conventional serology (FC-ALTA) (Krettli, 2009), especially in hosts treated with chronic infections or mainly with later chronic infections when their affinity for the *T. cruzi* antigen is strongly higher.

The histopathological analysis in the acute phase of the infection revealed that cardiac inflammation in mice infected with TcII samples was higher and showed significant difference than the observed for TcVI. The positive impact of treatment was clearly observed in animals infected with TcII and TcVI samples in the reduction or elimination of parasitemia and inflammatory lesions, similarly to the verified by Oliveira-Silva et al. (2015) with others TcII samples of the same locality. No impact of nifurtimox treatment, neither reduction of cardiac lesions (inflammation and fibrosis), were observed during the chronic phase of infection.

In summary, *in vitro* essays (acellular and cellular cultures) TcII parasites showed always higher values for all parameters (growth and infectivity) than TcVI, followed by TcI. The same tendency was observed in mice infections regarding to parasitemia. An association between parasite genetic and heart lesions in mice model and human disease was verified with the TcII samples (452 and 728). Mice infected with these samples presented severe inflammatory lesions, as well as patients from whom these samples were isolated, which displayed severe clinical forms of the disease, cardiac and cardio-digestive, respectively. On the other hand, the TcII sample (501), isolated of a patient with the indeterminate clinical form of Chagas disease, despite had displayed higher parasitemia in mice, the inflammation in the heart was discreet, suggesting the important hole of this lesion on clinical Chagas disease evolution. Interestingly, the only TcVI sample which displayed low values for the same parameters evaluated caused in human the indeterminate form of the disease. The lack of correlation between mice infection evolution and human clinical evolution of the disease may be explained by the fact that we do not know if some patient has parasites of other DTU in the tissues distinct of the sample isolated, taking into account the clonal histotrophic model for *T. cruzi* (Macedo et al., 2004).

Although the clonal theory accepted for *T. cruzi* implies in correlation between genetic distance and the biological characteristics of the parasites populations of this specie, the occurrence of genetic exchange (hybridization phenomenon) may not be excluded. Consequently the tentative of to demonstrate these correlations is easier when the parasites are of the DTUs TcI and TcII. In fact, this was also verified in this manuscript and in others already published by our team (Lana et al., 1998; Toledo et al., 2002, 2003) and others (Andrade and Magalhães, 1997). However, when parasites of hybrid DTUs are considerate the correlations are not so clear and may be interpreted as consequence of the genetic background of these parasites which involve genetic components of two DTUs: TcI and TcII for TcV and TcVI or TcIII and TcIV in the case of TcII and TcIII.

Even considering the few number of *T. cruzi* samples, and the impossibility of to compare *in vivo* all samples, an apparent or discreet correlation was verified regarding parasite genetic and its general biological characteristics. However, apparently human disease evolution is not exclusively associated to parasite genetics, but also to its ability in develop histopathological lesions in the host, what in turn, is dependent to its genetic and immune response (Tibayrenc and Ayala, 2015). Probably the comparison of more samples of distinct DTUs could to show a stronger association among all aspects as verified by Andrade and Magalhães (1996) and Toledo et al. (2002, 2003) in mice. However this study is still valid and is the first that tried verifying correlation between four aspects in parallel (*T. cruzi* genetics, its biology *in vitro* and *in vivo* and the clinical aspects of the disease in patients from whom the parasites were isolated).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Maykon Tavares Oliveira: involved with all laboratorial activities related to the maintenance of parasites in acellular media, cellular media in a murine model; Ana Paula Vieira de Oliveira: undergraduate research student, with all laboratorial activities; Renata Tupinambá Branquinho: assistance in the performance of parasitaemia in mice and help in statistical analysis; Gláucia Diniz Aléssio: responsible for conducting the FC-ALTA; Carlos Geraldo Campos Mello: responsible for carrying out and interpretation of the ELISA data; Nívea Carolina Nogueira de Paiva: responsible for carrying out analysis and histopathology in cardiac tissue of animals; Cláudia Martins Carneiro: responsible for carrying out analysis and histopathology in cardiac tissue of animals; Max Jean de Ornela Toledo: collaborator responsible for the analysis of biological parameters in a murine model; Willian de Castro Borges:

co-leader of the project; Olindo de Assis Martins-Filho: coordinator flow cytometry platform and collaborator in the analysis of results FC-ALTA; Marta de Lana: coordinator of the study and collaborations.

All authors have participated in this study and agree with the final version of the manuscript.

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