Am. J. Trop. Med. Hyg., 98(2), 2018, pp. 453–463
doi:10.4269/ajtmh.16-0200

Biological and Molecular Characterization of *Trypanosoma cruzi* Strains from Four States of Brazil

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Abstract. Chagas disease affects between six and seven million people. Its etiological agent, *Trypanosoma cruzi*, is classified into six discrete typing units (DTUs). The biological study of 11 *T. cruzi* strains presented here included four parameters: growth kinetics, parasitemia curves, rate of macrophage infection, and serology to evaluate IgM, total IgG, IgG1, IgG2a, and IgG3. Sequencing of small subunit of ribosomal RNA (SSU rRNA)was performed and the *T. cruzi* strains were classified into three DTUs. When their growth in liver infusion tryptose medium was represented in curves, differences among the strains could be noted. The parasitemia profile varied among the strains from the TcI, TcII, and TcIII groups, and the 11 *T. cruzi* strains produced distinct parasitemia levels in infected BALB/c. The TcI group presented the highest rate of macrophage infection by amastigotes, followed by TcII and TcIII. Reactivity to immunoglobulins was observed in the TcI, TcII, and TcIII; all the animals infected with the different strains of *T. cruzi* showed anti-*T. cruzi* antibodies. The molecular study presented here resulted in the classification of the *T. cruzi* strains into the TcI (Bolivia, T lenti, Tm, SC90); TcII (Famema, SC96, SI8, Y); and TcIII (QMM3, QMM5, SI5) groups. These biological and molecular results from 11 *T. cruzi* strains clarified the factors involved in the biology of the parasite and its hosts. The collection of triatomine (vector) species, and the study of geographic distribution, as well as biological and molecular characterization of the parasite, will contribute to the reporting and surveillance measures in Brazilian states.

INTRODUCTION

Chagas disease presents an annual incidence of 28,000 cases in the Americas. It is estimated that 65,000,000 people live in areas of risk.[1] The study of the biochemical, genetic, and biological markers in *Trypanosoma cruzi* has allowed for the separation of the parasite into six discrete typing unit (DTU) groups known as TcI to TcVI.[2,3]

The genus *Triatoma* is of epidemiological importance because most vector species transferring *T. cruzi* to humans belong to this genus, an example is *Triatoma infestans*, considered the main domiciled species.[4,5] In Latin American countries, Chagas disease is considered an important cause of death and affects between six and seven million people.[6] Vector-borne transmission of *T. cruzi* may occur through one of the 148 triatomine species.[7] For this reason, epidemiological studies are performed in both endemic and nonendemic areas, and triatomines are also captured for analysis.[8–14]

Many studies have been performed in an attempt to describe the genetic structure of the parasites.[15] These efforts have included the sequencing of the genome of *T. cruzi* (CL Brener), which was published in conjunction with the genome sequences of *Leishmania major* and *Trypanosoma brucei*. The current classification of *T. cruzi* proposes a triple assay using rDNA polymerase chain reaction (PCR)[16] and PCR–restriction fragment length polymorphism of the HSP60 and GPI loci[17] to type the six *T. cruzi* DTUs[2,3] and suggests that the TcI and TcII groups are molecularly more divergent. The phylogenetic relationship between TcIII and TcIV may be interpreted as a hybridization event between the TcI and TcII groups ("Two-Hybridization" model) and in another model TcIII is also ancestral ("Three Ancestor model").[18]

Both Two-Hybridization model[17] and Three Ancestor model[18] incorporate two hybridization events. In the Three Ancestor model, two recent genetic exchange events between TcII and TcIII yield TcV and TcVI. The Two-Hybridization model invokes one ancient genetic exchange event between TcI and TcII, with loss of heterozygosity among progeny to produce TcIII and TcIV, followed by a second more recent hybridization event between TcII and TcIII to yield both TcV and TcVI.[18]

The major difference between the Two-Hybridization and the Three Ancestor models is, therefore, whether TcV and TcVI are progeny from a single hybridization event incorporating TcI alleles acquired via TcIII[17] or progeny of two hybridization events excluding TcI.[19] The TcI group is more abundant and widespread; there have been recent attempts to characterize it into sub-DTUs.[20–24] The ability to isolate multiple *T. cruzi* clones from a given host explains the existence of intrapopulation patterns within the TcI strains.[25] The TcI group is of ecological and evolutionary importance, particularly in terms of the sylvatic cycle of infection.[26]

TcII is found predominantly in the southern and central regions of South America, but its true extent is not yet

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clear. It has been isolated mostly from domestic transmission cycles. The natural hosts and vectors of TcII have proven elusive and most of the reported isolations have been made in the remaining fragments of the Atlantic forest of Brazil, from primates and sporadically from other mammalian species.[27–29] The divergence date between TcI and TcII is ill defined; it is estimated as being between 88 and 37 million years ago, based on small subunit rDNA,[30,31] and between 16 and 3 million years ago, based on dihydrofolate reductase–thymidylate synthase and trypanothione reductase genes.[32]

Since TcIII is present in the domestic cycle, this group is responsible for human cases of Chagas disease, as has already been described.[33] TcIII participation in sylvatic cycles has also been described in different biomes and involves infection of several wild hosts, ranging from bats to carnivores.[34] TcIV shows a similar pattern of distribution in South America to TcIII. Unlike TcIII, TcIV occurs fairly frequently in humans and is a secondary cause of Chagas disease in Venezuela.[35] Further research is required to understand the history of TcIV and these complex ecological associations.[18]

TcV and TcVI are two similar hybrid DTUs associated with Chagas disease in southern and central South America. The understanding of the ecology of TcII, TcV, and TcVI is as yet vulnerable to the limited sampling of sylvatic hosts and vectors. The paradigms may change. It has been suggested that TcII, TcV, and TcVI are more widespread geographically than is currently acknowledge and might be found much further north.[36] A recent study demonstrated the finding of TcII infecting triatomine bugs and mammals in two different areas of the Brazilian Amazon, a biome always before quoted as free from TcII.[37] If the known TcV and TcVI hybrids are also found in Central and North America, it will most likely imply recent migration with humans or other carriers; if genetically distinct TcII/TcIII hybrids are observed, hybridization may be an ongoing phenomenon where such mixed infections occur.[18]

The biological and molecular characterization of *T. cruzi* strains aid in the understanding of parasite–host interactions. This elucidation is seen in the study by Zingales et al.,[18] who discuss associations between vectors, breeding sites, hosts, and the six distinct *T. cruzi* groups (DTUs). Although the division of *T. cruzi* into six groups (TcI–TcVI) makes it easier to understand the diversity of this protozoan (a diversity that, it is important to mention, was already noted in the description by Chagas in 1909[38]), the artificial nature of this classification became apparent in the study by Pena et al.[39] in which mixed TcI/TcII *T. cruzi* populations that had been isolated from *Triatoma tibiamaculata* were identified.

This investigation was conducted to provide a biological and molecular characterization of *T. cruzi* isolated from triatomine specimens collected in Brazilian states, as the understanding of the *T. cruzi* DTUs and their epidemiological implications could provide new insights to guide research and interventions against this devastating infectious disease.

To clarify the biological and molecular parameters of *T. cruzi* using in vivo and in vitro studies, this work presents the parasite's rate of macrophage infection, growth kinetics based on liver infusion tryptose (LIT) culture, parasitemia curves using BALB/c mice and reactivity to immunoglobulins, and the phylogeny of 11 strains (TcI, TcII, and TcIII) isolated in the Brazilian states of Bahia (*Triatoma sordida*/*Triatoma melanocephala*/*Triatoma lenti*), Rio Grande do Sul (*Triatoma rubrovaria*), Santa Catarina (*Didelphis aurita*/*Homo sapiens*), and São Paulo (*H. sapiens*). The study of strains isolated from the five species of triatomines belonging to the genus *Triatoma* is needed and would complement data on the biological and molecular characterization of the 11 strains of *T. cruzi* belonging to DTUs TcI, TcII, and TcIII. The results achieved with this work can be used to detect ecological factors associated with *T. cruzi*, which are part of a complex epidemiological context, and help to increase the knowledge of Chagas disease in the regions from which the triatomines were collected.

MATERIALS AND METHODS

Strains of *T. cruzi*. A total of 11 strains were characterized in the present study, six strains were isolated from the posterior intestine of triatomine species[12]: *Triatoma melanocephala* from Bahia-BR—Tm (TcI); *T. lenti* from Bahia-BR—T lenti strain (TcI); *T. sordida* from Bahia-BR—SI5 (TcIII) and SI8 (TcII) strains; and *T. rubrovaria* from Rio Grande do Sul-BR—QMM3 and QMM5 strains (TcIII). Five further strains of *T. cruzi* were included in the biological assays as reference strains: Bolivia[40] from Vitichi-BO (*T. infestans*—TcI); Famema[41] from São Paulo-BR (*H. sapiens*—TcII); SC90[42] from Santa Catarina-BR (*D. aurita*—TcI); SC96[42] from Santa Catarina-BR (*H. sapiens*—TcII); and Y[43] from São Paulo-BR (*H. sapiens*—TcII). A total of 44 sequences were studied in the phylogenetic analysis. Sequences from 26 *T. cruzi* isolates and three sequences of *Trypanosoma cruzi marinkellei* from GenBank were included in the phylogenetic analysis (with the accession numbers shown in Table 1). The 18 sequences that were determined in this study and deposited in GenBank are underlined (Table 1).

Small subunit rRNA gene amplification (V7V8 region) of *T. cruzi*. DNA was extracted according to the methods of Sambrook et al.,[44] with modifications. The amount of DNA was estimated through comparisons to known standards in 1% (w/v) agarose gel stained with GelRed®, as well as through the use of spectrophotometry. The PCRs contained 100 ng of genomic DNA, 100 ng of each primer, 200 mM of each dNTP, 5 µL of a buffer solution (Tris-HCl [pH 8.4], 500 mM KCl, and 1.5 mM $MgCl_2$), 2.5 U of *Taq* DNA polymerase, and double distilled deionized and autoclaved water (in a final volume of 50 µL). The amplification cycle and the annealing temperatures were defined according to the oligonucleotide primers used, which were 609F (5′-GAT CCG CGG TAA TTC CAG C-3′) and 706R (5′-TTG AGG TTA CAG TCT CAG-3′).

Nucleotide sequencing. The DNA fragments that were amplified using PCR were purified and then underwent sequencing reactions using a Big Dye Terminator kit (Perkin Elmer; Applied Biosystems Inc., Austin, TX) according to the manufacturer's instructions. The reactions were performed with an initial cycle of 1 minute at 96°C, followed by 30 cycles of 15 seconds at 96°C, 15 seconds at 50°C, 4 minutes at 60°C. The following oligonucleotide primers were used: 1156F (5′-CGT ACT GGT GCG TCA AGA GG-3′); 1156R (5′-CCT CTG ACG CAC CAG TCA G-3′); 609F (5′-GAT CCG CGG TAA TTC CAG C-3′); and 706R (5′-TTG AGG TTA CAG TCT CAG-3′).

TABLE 1

Isolates of *Trypanosoma cruzi*, host and geographical origin, lineages, and sequences of SSU rDNA characterized in this study

Isolate	Host origin		Geographic Origin (state)		DTU*	GenBank accession number† V7V8 SSU rRNA
Trypanosoma cruzi						
Tm	Triatomine	*Triatoma melanocephala*	Bahia	BR	TcI	xxxxxx
Bolivia cl3	Triatomine	*Triatoma infestans*	Vitichi	BO	TcI	xxxxxx
Bolivia cl4	Triatomine	*T. infestans*	Vitichi	BO	TcI	xxxxxx
SC90	Opossum	*Didelphis aurita*	Santa Catarina	BR	TcI	xxxxxx
G	Opossum	*Didelphis marsupialis*	Amazonas	BR	TcI	AF239981
TCC45	Opossum	*D. aurita*	São Paulo	BR	TcI	FJ183394
Sylvio X10	Human	*Homo sapiens*	Para	BR	TcI	AF303659
TCC269	Wild primate	*Saguinus midas*	Amazonas	BR	TcI	EU755221
T lenti	Triatomine	*Triatoma lenti*	Bahia	BR	TcI	xxxxxx
SC96 cl3	Human	*H. sapiens*	Santa Catarina	BR	TcII	xxxxxx
SC96 cl4	Human	*H. sapiens*	Santa Catarina	BR	TcII	xxxxxx
SI8 cl1	Triatomine	*Triatoma sordida*	Bahia	BR	TcII	xxxxxx
SI7	Triatomine	*T. sordida*	Bahia	BR	TcII	xxxxxx
FAMEMA	Human	*H. sapiens*	Sao Paulo	BR	TcII	xxxxxx
Esmeraldo clone 3	Human	*H. sapiens*	Bahia	BR	TcII	AY785564
TCC139	Opossum	*D. aurita*	São Paulo	BR	TcII	FJ001616
Y	Human	*H. sapiens*	São Paulo	BR	TcII	AF301912
SIGR3 cl1	Cat	*Felis silvestris catus*	Bahia	BR	TcII	xxxxxx
QMM3	Triatomine	*Triatoma rubrovaria*	Rio Grande do Sul	BR	TcIII	Xxxxxx/xxxxxx
QMM5	Triatomine	*T. rubrovaria*	Rio Grande do Sul	BR	TcIII	Xxxxxx/xxxxxx
QMM12	Triatomine	*T. rubrovaria*	Rio Grande do Sul	BR	TcIII	xxxxxx
SI5 cl1	Triatomine	*T. sordida*	Bahia	BR	TcIII	Xxxxxx/xxxxxx/xxxxxx
Arma13 cl1	Armadillo	*Dasypus novemcinctus*	Boqueron	PY	TcIII	FJ549385
TCC2557	Bat	*Phyllostomus hastatus*	São Paulo	BR	TcII	KT305894
TCC863	Armadillo	*Euphractus sexcinctus*	Rio Grande do Norte	BR	TcIII	FJ549376
MT3869	Human	*H. sapiens*	Amazonas	BR	TcIII	AF303660
MT3663	Triatomine	*Panstrongylus geniculatus*	Amazonas	BR	TcIII	AF288660
Can III	Human	*H. sapiens*	Para	BR	TcIV	AJ009148
TCC206	Carnivore	*Nasua nasua*	Para	BR	TcIV	FJ555615
TCC1441	Human	*H. sapiens*	Para	BR	TcIV	EU755247
TCC1446	Human	*H. sapiens*	Para	BR	TcIV	EU755248
M6241 cl6	Human	*H. sapiens*	Para	BR	TcIII	AY785578
Sc43 cl1	Triatomine	*T. infestans*	Santa Cruz	BO	TcV	AF232214
TCC186	Triatomine	*T. infestans*	–	BO	TcV	FJ001630
CL Brener	Triatomine	*T. infestans*	Rio Grande do Sul	BR	TcVI	AF245383
TCC1122	Bat	*Myotis albescens*	São Paulo	BR	Tcbat	FJ001628
TCC294	Bat	*Myotis levis*	São Paulo	BR	Tcbat	FJ001634
TCC1994	Bat	*M. levis*	São Paulo	BR	Tcbat	FJ900241
TCC597	Bat	*Myotis nigricans*	Mato Grosso do Sul	BR	Tcbat	FJ001623
NR cl3	Human	*H. sapiens*	Salvador	CL	TcV	AF228685
Trypanosoma cruzi marinkellei						
TCC421	Bat	*Phyllostomus discolor*	Amazonas	BR	–	FJ001637
TCC424	Bat	*P. discolor*	Amazonas	BR	–	FJ001639
TCC501	Bat	*Carollia perspicillata*	Rondonia	BR	–	FJ001665

BO = Bolivia; BR = Brazil; CL = Chile; DTU = discrete typing unit; PY = Paraguay; SSU = small subunit.
* Lineages determined based on mini-exon markers and phylogenetic analyses inferred in this study.[48]
† Sequences determined in this study and deposited in GenBank are underlined.

Nucleotide sequencing alignment. The sequencing chromatograms were analyzed with the Seqman-DNAstar program (DNASTAR Inc., Madison, Wisconsin).[45] Both the nucleotide sequences determined in this study and those obtained from GenBank (http://www.ncbi.nlm.nih.gov/) were aligned using the Clustal X program.[46] The nucleotide alignments were manually adjusted in the GeneDoc program, version 2.7.000.[45]

Phylogenetic analyses. Phylogenetic interferences were determined using the maximum likelihood (ML) method and Bayesian analysis. The tree was developed using the PAUP program, version 4.0b10[47] through the heuristic search with 100 random addition replicates of the terminals, followed by branch breaking. One hundred replicates of the bootstrap support analyses were performed with the same parameters used in the search.

The ML analyses were performed using the RAxML program, version 7.0.4.[48] A total of 500 replicates were used using the generalized time reversible analysis as a substitution model, and four gamma categories, and diagrams obtained through parsimony were used as initial trees. The parameters of the substitution model were estimated during the search. Branch support was estimated using 500 bootstrap replicates in the RAxML program.

The Bayesian analyses were performed in the MrBayes program, version 3.1.2.[49] A total of 500,000 generations were used using generalized time reversible analysis as the substitution model, and four gamma categories, plus a proportion of invariant sites. Only the diagrams obtained from the last 75 replicates were used to build the final cluster tree. The genealogies of the nucleotide sequences were inferred using network analysis in the Splitstree program, version 4.11.3.[50] The NeighborNet method was used, and the support values were estimated through the completion of the 100 bootstrap replicas.

Parasitemia curve. *Trypanosoma cruzi* trypomastigote counts were performed based on the method of Brener.[51] To study the parasitemia curve, BALB/c mice—five animals for each group (22-day-old males weighing between 25 and 30 g)—were intraperitoneally inoculated with 5×10^3 trypomastigote forms of *T. cruzi*. To establish the infection pattern, 5 µL of blood obtained from the tails of the mice was examined microscopically and the number of forms was established. Counts were performed in all the strains studied on alternate days, starting from the second day after the initial inoculation until the 60th day of the infection. The number of animals that died during the course of the infection was observed on a daily basis. The animals were maintained under temperature- and light-controlled conditions. The experiments were approved by the Research Ethics Committee of the School of Pharmaceutical Sciences within São Paulo State University, Araraquara, Brazil (CEUA/FCF/CAr no 13/2012).

Growth kinetics. *Trypanosoma cruzi* epimastigote growth dynamics were studied using the Bolivia, Famema, QMM_3, QMM_5, SC96, SC90, SI_5, SI_8, TI, Tm, and Y strains. The study was performed by inoculating 5×10^6 parasites per mL in 5 mL of LIT medium. Cell counts were measured five times over 10 days in a Neubauer chamber using an optic microscope.

***Trypanosoma cruzi* assay in peritoneal macrophages.** The invasion assay was performed according to Muelas-Serrano et al.[52] The macrophages were grown at 37°C in Roswell Park Memorial Institute media with 5% CO_2. For the assay, 5×10^5 cells were seeded in 24-well plates. The cells were incubated at 37°C for 4 hours and then infected with 5×10^6 *T. cruzi* trypomastigotes. (These trypomastigotes had been obtained in stationary phase, washed in phosphate-buffered saline (PBS), and the concentration adjusted using a Neubauer chamber.) At 24 and 72 hours after infection, the cells were washed in PBS, fixed in methanol, and stained using Giemsa. The rate of infection was determined by counting 200 macrophages that contained internalized amastigote forms. The counts were repeated three times and the parasite duplication time of the 11 *T. cruzi* strains in macrophages was determined using the formula reported by Jawetz et al.[53]

$$\mathrm{dt} = \frac{\ln 2(t_1 - t_0)}{2,3 \log(N_1/N_0)}$$

where dt = doubling time; t_1 = 72 hours; t_0 = 24 hours; N_1 = mean number of parasites per infected cell at 72 hours of infection; and N_0 = at 24 hours of infection.

Determination of the presence of anti-*T. cruzi* antibodies. An enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of anti-*T. cruzi* antibodies (IgM, IgG, IgG1, IgG2a, and IgG3) in the serum of mice infected with the TcI, TcII, and TcIII genotypes of *T. cruzi*. Mice ($N = 3$ per strain) were injected intraperitoneally with 1×10^5 trypomastigote forms of *T. cruzi*, strain Bolívia, Y, and QMM5, and killed at 30, 55, and 70 days after infection. Blood samples were collected by cardiac puncture, without anticoagulant, and the sera obtained were stored at –20°C. Healthy mice were used as a negative control ($N = 3$). For the positive control, a polyclonal anti-*T. cruzi* serum was produced by inoculation of a crude antigen extract of *T. cruzi* epimastigotes. CD1 mice were inoculated with 100 µL of the *T. cruzi* extracts by the intraperitoneal route. After the immunization period, the animals were euthanized, and blood samples were then collected for serum fractionation, and stored at –20°C. To determine the presence of anti-*T. cruzi* antibodies, 100 ng/mL of the *T. cruzi* epimastigote antigen was diluted in a bicarbonate buffer (0.1 M and pH 8.5) and incubated overnight at 5°C in microplates. The microplates were washed with wash buffer (PBS–Tween-20, 0.05% v/v), and incubated with a Bovine Serum Albumin blocking buffer (PBS–BSA–Tween-20, 0.05%) for 1 hour, at which point they were washed again. The plates were incubated for 1 hour with 100 µL diluted serum (1:400) and washed five times with the wash buffer. To determine the presence of anti-*T. cruzi* IgG antibodies, anti-IgG secondary antibody solution conjugated with enzyme horseradish peroxidase (HRP) 1:4,000 anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) was added to the plates and incubated for 1 hour. The other antibody subclasses were determined with the following conjugated antibodies: IgG1 (50 ng of anti-mouse IgG1:HRP; AbD Serotec); IgG2a (25 ng anti-mouse IgG2a:HRP; AbD Serotec); IgG3 (25 ng anti-mouse IgG3:HRP; AbD Serotec); and IgM (1:400 anti-mouse IgM:HRP; Sigma-Aldrich). Next, five washes were performed and the microplate was incubated with a substrate solution for 30 minutes (10 mL of citrate buffer, 10 mg of o-phenylenediamine, and 5 µL of hydrogen peroxide 3% [v/v]). In addition, 4 N sulfuric acid was used to stop the reaction, and absorbance was measured at 490 nm. The assay was performed in duplicate and the optical density data were presented as means and standard deviations, using the software Instat (Graphpad, San Diego, CA). The analysis of variance for all the data was carried out by two-way analysis of variance.

RESULTS

Molecular characterization. In this work, *T. cruzi* strains were separated into TcI (Bolivia, Tm, T lenti, and SC90); TcII (Y, FAMEMA, SI8, and SC96); and TcIII (SI5, QMM3, and QMM5). The origins, hosts, and DTUs of the *T. cruzi* strains are reported in Table 1, and the phylogenetic relationships among the 11 strains are shown in Figure 1. In all analyses (ML and Bayesian), *T. cruzi* formed a monophyletic assemblage.

Parasitemia profile. It was found that the 11 *T. cruzi* strains resulted in distinct parasitemia levels in infected BALB/c mice, despite the fact that the inoculum (5×10^3) had been standardized in the TcI, TcII, and TcIII groups. The parasitemia profile of the TcI, TcII, and TcIII groups during the acute phase of the experimental infection by *T. cruzi* is presented in Figure 2.

In the TcI group (Bolívia, SC90, T lenti, and Tm), subpatent infection was observed for the SC90 and T lenti strains, although *T. cruzi* was not found in the bloodstream. In the case of the Tm strain, peak parasitemia occurred around the 15th day, with a mortality rate of 20% .

In the TcII group (Famema, SC96, SI8, and Y), the Famema strain had more trypomastigote forms and a parasitemic peak around the 27th day. The TcIII group of *T. cruzi*, represented by SI5, QMM3, and QMM5, showed the SI5 strain with more trypomastigotes and a parasitemic peak on the 52th day. The SI5 (TcIII) and SI8 (TcII) *T. cruzi* strains presented differences in the prepatent period, in the

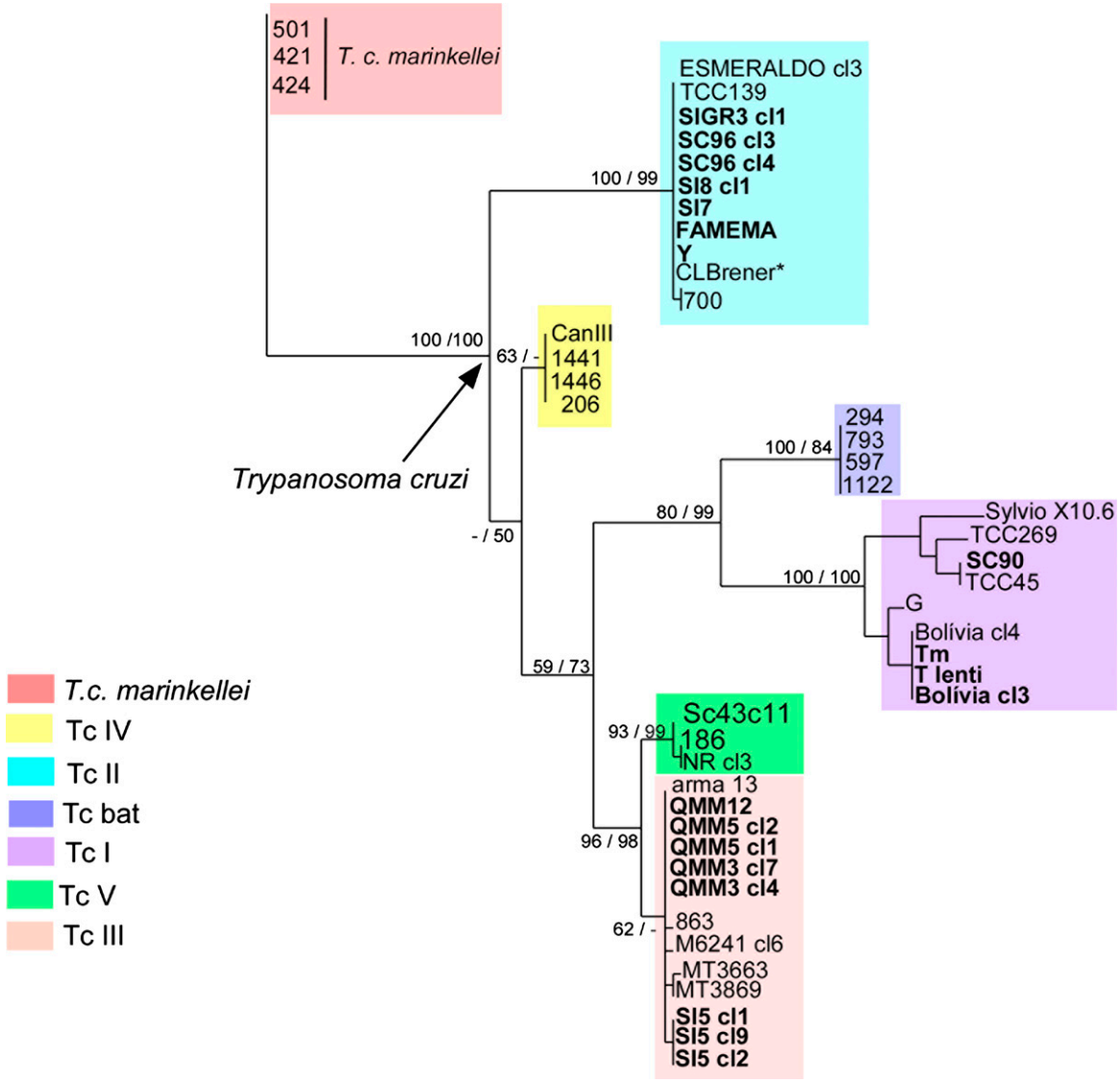

FIGURE 1. Phylogenetic relationships between *Trypanosoma cruzi* strains based on small subunit rRNA sequences. The numbers correspond to the bootstrap values derived from the 500 replicas in the maximum likelihood and Bayesian analyses. This figure appears in color at www.ajtmh.org.

peak parasitemia levels, and in the decreases in blood trypomastigote populations, although the SI5 and SI8 have been isolated from *T. sordida*. After that period, the number of blood trypomastigotes started to decrease, with trypomastigotes disappearing from the bloodstream around the 60th day of the infection.

Biological variability of *T. cruzi* strains in LIT medium. When *T. cruzi* growth in LIT medium was represented by curves, differences among the strains could be noted (Figure 3). Maximum parasite multiplication occurred at around 5–10 days (10.9×10^6–8.7×10^6 parasites) for the TcI group, 6–9 days (13.2×10^6–14.8×10^6 parasites) for the TcII group, and 7–8 days (13.2×10^6–11×10^6 parasites) for the TcIII group. Together, these results are suggestive that the maintenance of populations of *T. cruzi* may be related to intrinsic characteristics of the parasite, such as its infection ability.

Infection rate of different strains of *T. cruzi* in peritoneal macrophages. The mean amastigote counts of the 11 *T. cruzi* strains in 200 peritoneal macrophages are represented in Figure 4. The *T. cruzi* groups were separated into TcI (Bolivia, Tm, T lenti, and SC90); TcII (Y, FAMEMA, SI8, and SC96); and TcIII (SI5, QMM3, and QMM5). The TcI group presented the following values for 24 and 72 hours of infection: 479–751 amastigotes/200 macrophages (Bolívia); 453–858 amastigotes/200 macrophages (Tm); 391–702 amastigotes/200 macrophages (T lenti); and 230–501 amastigotes/200 macrophages (Sc90). The TcII group had the values: 415–702 amastigotes/200 macrophages (Y); 490–702 amastigotes/200 macrophages (FAMEMA); 392–822 amastigotes/200 macrophages (SI8); and 611–799 amastigotes/200 macrophages (SC96). The TcIII group had the values: 546–682 amastigotes/200 macrophages (SI5); 388–610 amastigotes/200 macrophages (QMM3); and 335–603 amastigotes/200 macrophages (QMM5). In *T. cruzi*, the times required for the multiplication of amastigote forms were TcI, between 1.7 and 3 days; TcII, between 1.8 and 5.1 days; TcIII, between 2.3 and 6.2 days. So the TcI group

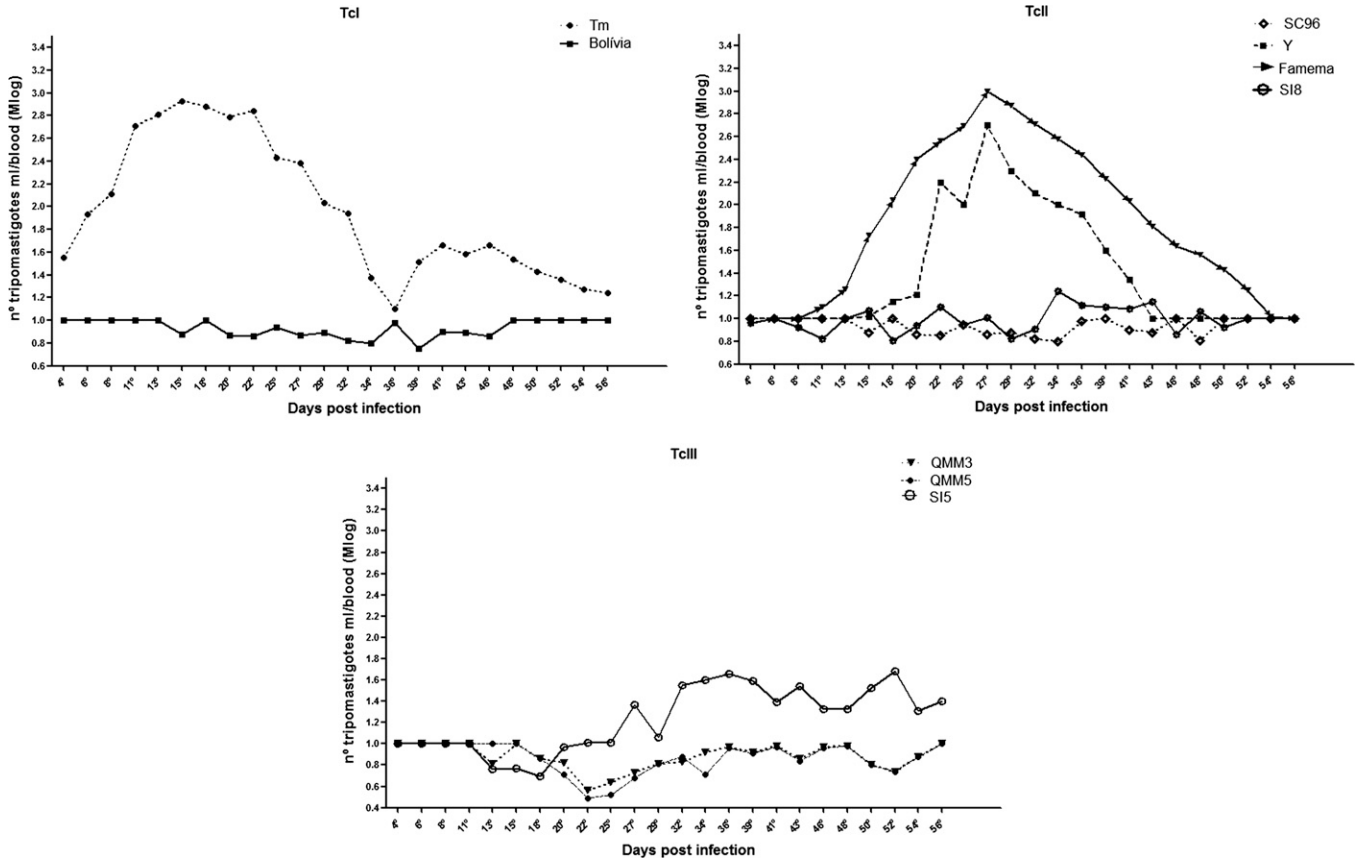

FIGURE 2. Parasitemia curve of *T. cruzi* belonging to the TcI, TcII, and TcIII groups in BALB/c mice over 60 days.

showed the least time required for the multiplication of the parasite, followed by TcII and TcIII.

The strains with the highest rates of infection in macrophages were isolated from *T. melanocephala* (Tm) and *T. sordida* (SI5/SI8). The insects were collected in the state of Bahia, Brazil, data reinforcing the parasite–host–environment association, as well as the adaptation of *T. cruzi* TcI, TcII, and TcIII to their vertebrate or invertebrate host.

Determination of anti-*T. cruzi* antibodies in mice infected with different strains of *T. cruzi*. All the animals infected with different strains of *T. cruzi* showed anti-*T. cruzi* antibodies (Figures 5 and 6). There was a decreased production of anti-*T. cruzi* IgM antibody in all groups 30 days after infection, due to the time-dependent decrease of infection (Figure 5A). The IgM decrease 30 and 70 days after infection was significantly different in Y and QMM5 strain groups, using Tukey's multiple comparison test ($P < 0.0001$). The Y strain triggered increased IgM production and QMM5 less antibody production. A progressive increase of the production of anti-*T. cruzi* total IgG was verified at 30, 55, and 70 days postinfection (Figure 5B), with significant differences in all groups ($P < 0.001$). The comparison among the groups demonstrated more pronounced differences compared with Y and QMM5 strains (Figure 5B). The analysis of the anti-*T. cruzi* antibody subclasses IgG1, IgG2a, and IgG3 demonstrated an increase of the titers followed by an increasing time of infection (Figure 6), which was most significant at 70 days postinfection ($P < 0.0001$). The Y strain showed higher titers of anti-*T. cruzi* IgG1 (Figure 6A), with significant differences compared with strain QMM5 (P value < 0.01) and no differences compared with Bolivia. Seventy days after infection, IgG2a and IgG3 antibody production was significantly more pronounced compared with production at 30 days after infection ($P < 0.0001$). The Y strain demonstrated higher titers compared with the other strains at 70 days after infection (Figure 6B and C). As shown in Figure 6C, although there was an increase over the duration of the infection, the titers of IgG3 showed it was virtually absent from or only present at very low levels in the group infected with strain QMM5 compared with the other groups infected with strains Bolivia and Y ($P < 0.0001$).

DISCUSSION

Molecular characteristics of the *T. cruzi* parasite, such as the diversity of the DTUs, can explain the peculiarities of Chagas disease, as the inherent characteristics of the parasite result in different clinical manifestations.[54] The results of this study reflect *T. cruzi* diversity, which was first mentioned by Chagas.[38]

The association between the TcI group (Tm, T lenti, and SC90) and wild reservoir hosts, such as *T. melanocephala* and *T. lenti*, is reported for the first time herein, since no studies were found in the literature. The data from Câmara et al.[55] showed the interaction between TcII and humans, and the involvement of the TcIII group in the wild environment was also considered in their work. The QMM3 and QMM5 strains isolated from *T. rubrovaria* were placed in the TcIII group; therefore, given this species' wide range in the Brazilian state of Rio Grande do Sul, it must still be monitored because the species has the ability to spread into domestic environments.[13]

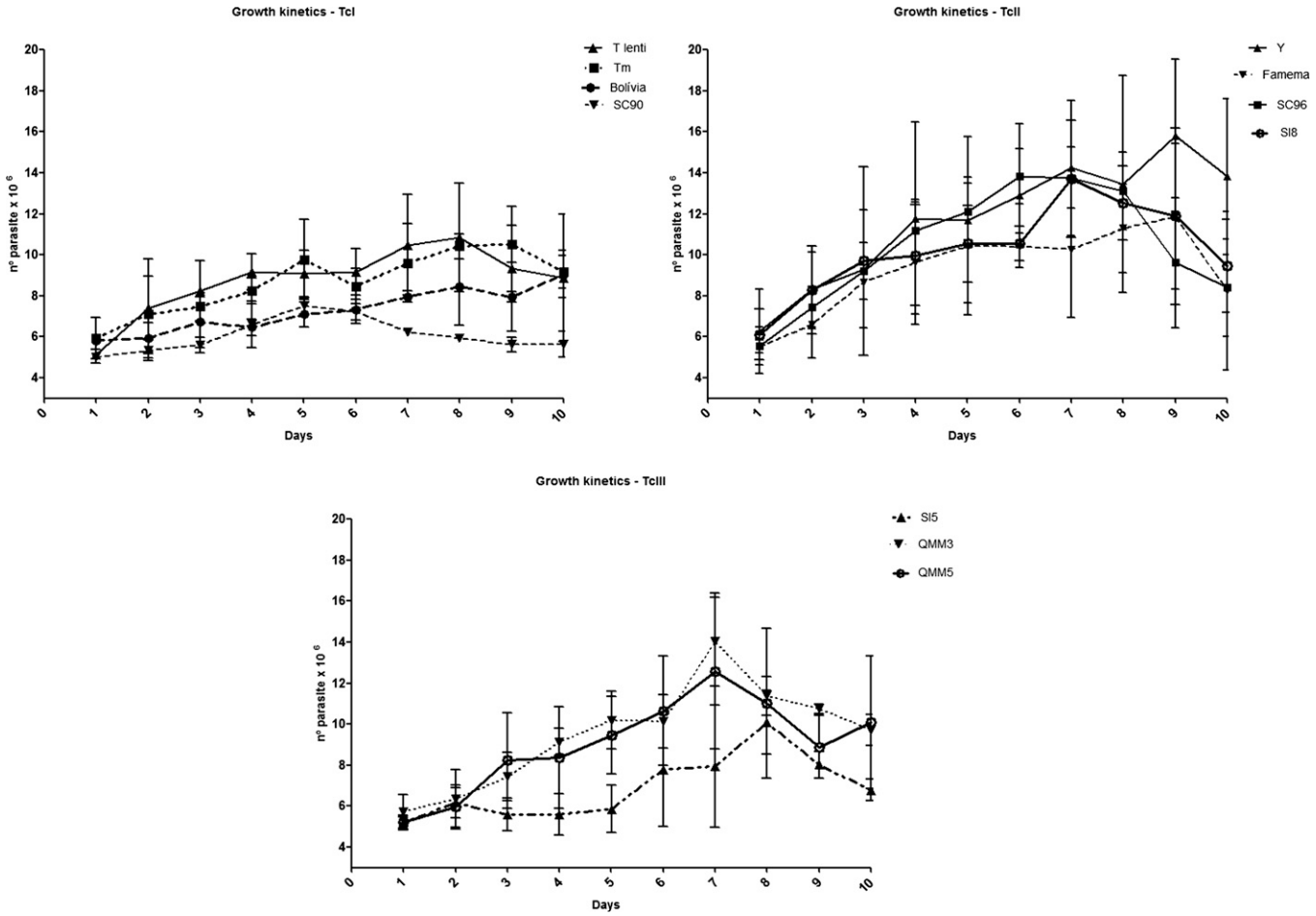

FIGURE 3. Growth kinetics of *Trypanosoma cruzi* (TcI, TcII, and TcIII) based on liver infusion tryptose culture. The parasite counts were repeated four times over 10 days in a Neubauer chamber using an optic microscope.

In ML and Bayesian analyses, TcI, TcII, and TcIII were clearly separated and the distribution found herein is consistent with the literature.[18,22] The findings are indicative that TcII is phylogenetically separated from TcI. The TcIII group developed later and was the basis of the hybridization event in *T. cruzi*.[18] The TcI group was placed as a sister group to Tcbat,[56] which has been established as a *T. cruzi* ancestor, forming a monophyletic assemblage and

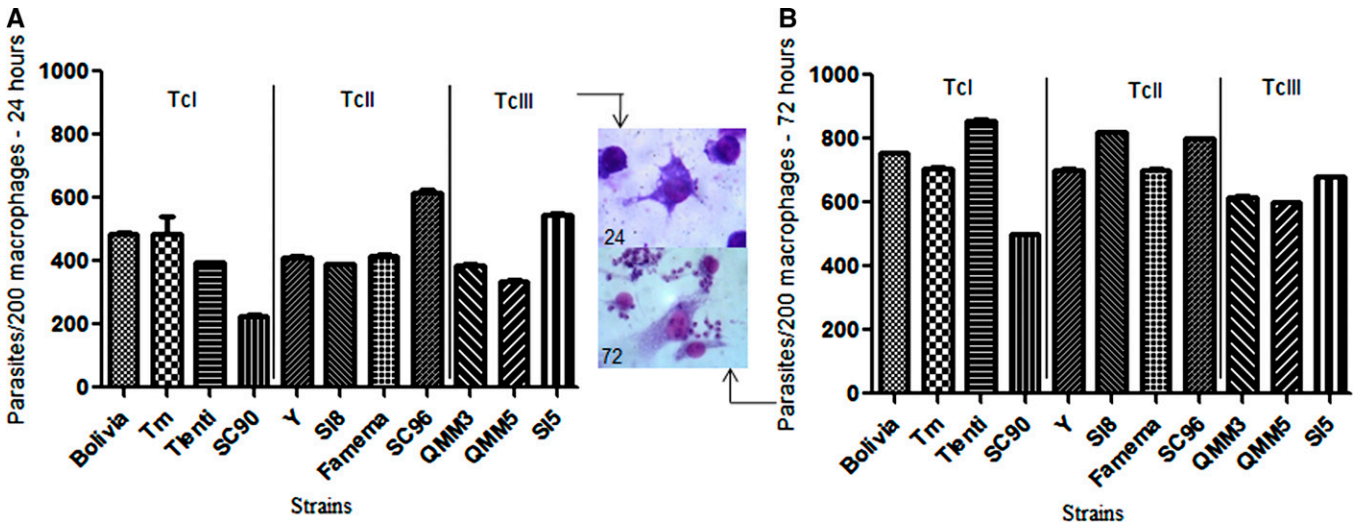

FIGURE 4. Mean number of *Trypanosoma cruzi* amastigote forms (TcI, TcII, and TcIII) seen in peritoneal macrophages after (**A**) 24 and (**B**) 72 hours of infection. The values represent the mean of the three tests performed. This figure appears in color at www.ajtmh.org.

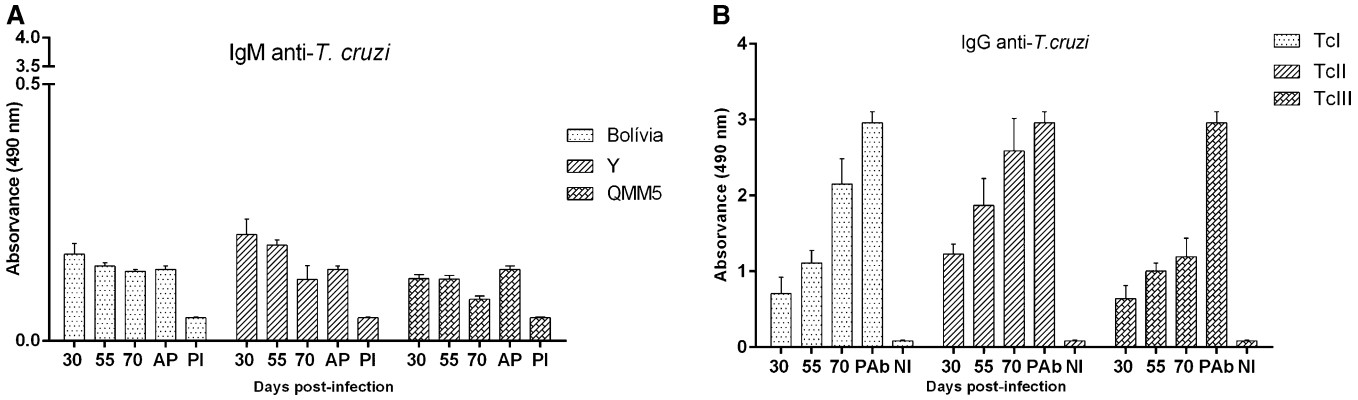

FIGURE 5. Determination of total (B) IgG and (A) IgM anti-*Trypanosoma cruzi* by enzyme-linked immunosorbent assay in serum from *Mus musculus* (BALB/c). Samples were collected 30, 55 and 70 days after infection with different strains of *T. cruzi* (Bolivia, Y and QMM5) belonging to TcI, TcII, and TcIII groups, respectively. Serum from healthy animals pre-immune (PI) were used as negative controls and polyclonal antiserum was used as positive control (AP). We used a dilution of 1:400 in the analysis of all samples. The analysis of variance for all the data was carried out by two-way analysis of variance.

clarified the evolutionary process of the taxon. As expected, the sequences of the hybrid TcIII strains were clustered very closely with strains of the TcV group, used as a control (Sc43c11, 186, NRc13).

Trypanosoma cruzi strains SI5 (Bahia—peridomestic), QMM3, and QMM5 (Rio Grande do Sul—wild environment) belonged to TcIII, highlighting the originality and importance of the work since triatomines collected in the peridomestic environment rarely belong to the TcIII group, because generally *T. cruzi*-TcIII is reported in the wild environment and only sporadically has been isolated from domestic transmission cycles. Generic identification methods based on

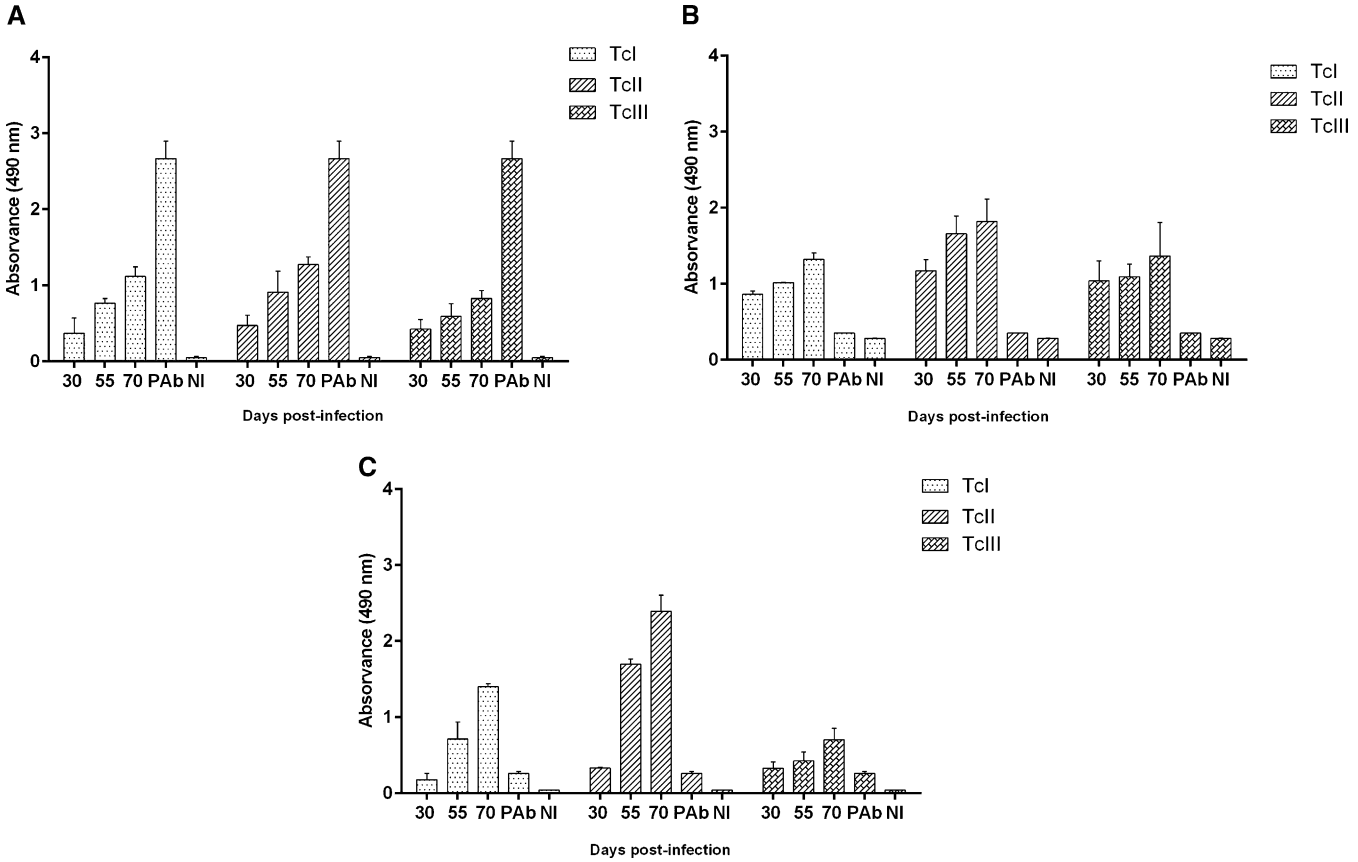

FIGURE 6. Determination of anti-*Trypanosoma cruzi* (A) IgG1, (B) IgG2a, and (C) IgG3 by enzyme-linked immunosorbent assay in serum from *Mus musculus* (BALB/c). Samples were collected 30, 55, and 70 days after infection with different strains of *T. cruzi* (Bolivia, Y, and QMM5) belonging to TcI, TcII, and TcIII groups, respectively. Serum from healthy animals and pre-immune (PI) serum were used as negative controls and polyclonal antiserum was used as a positive control (AP). We used a dilution of 1:400 in the analysis of all samples. The analysis of variance for all the data was carried out by two-way analysis of variance.

SSU rRNA sequences can be used to identify trypanosome species, as well as to infer phylogenetic relationships in trypanosomes and vectors.[57]

Some authors[58–60] have mentioned the variation of parasitemia in animals inoculated with *T. cruzi* strains, either for samples collected from humans, wild animals, or the vector itself. The parasitemia curves of the TcI (Bolivia, Tm, T lenti, and SC90), the TcII (Y, FAMEMA, SI8, and SC96), and the TcIII group (SI5, QMM3, and QMM5) showed differences in the prepatent period, stationary phase, and a decrease in parasitemia levels. Together these results show that the biological variability between TcI, TcII, and TcIII groups of *T. cruzi* can be explained by the host–parasite association and the geographical region that the Triatominae were collected from.

That variation is frequently seen when a new strain of the parasite is isolated and it is directly related to the stabilization of the relationship between the parasite and the vertebrate host.[61] Biological differences between the TcI and TcII groups were observed by Lisboa et al.[29] and in this work the population dynamics of the TcI, TcII, and TcIII groups presented culture differences, which may be linked to the parasite's adaptation to the axenic environment.

The establishment of *T. cruzi* infection depends on a series of events, involving interactions between the molecules of the parasite and the host.[62] In the case of macrophages, most parasites are internalized via the phagocytic mechanism, involving polymerization of actin filaments required for the formation of membrane projections.[63,64] This study devoted to new strains of *T. cruzi* aimed to create ideal conditions for the growth and differentiation of the parasite and as a result, the biological characteristics of *T. cruzi* became important in the interaction with the host cells during the infection process.

Despite advancements in the diagnosis of Chagas disease, new antigens are still needed.[65] During infection in the murine model, the humoral response to *T. cruzi* is mainly due to IgM and IgG2a antibodies for acute infection.[66] The low fraction of anti-*T. cruzi* IgM after 30 days of infection verified in this study was similar to results found in other studies, indicating that anti-*T. cruzi* IgM does not protect in the most advanced stage of infection.[67] The analysis in this study confirmed isotype switching of anti-*T. cruzi* IgM class to IgG.

The analysis of IgG antibodies, specifically anti-*T. cruzi* IgG1, in the present study demonstrated low levels during the acute phase, with the sharpest increase 70 days after infection, indicating a lack of protective ability of IgG1 in the acute phase. The results of this study are in accordance with studies reported in the literature, where it was found that during experimental infection, the immune response is a detectable high concentration of IgG2a anti-*T. cruzi* antibodies.[67,68] In this study, IgG1 and IgG3 antibodies were present only at very low levels during the acute phase 30 days after infection. Moreover, IgG2a is associated with protection and is considered more effective against *T. cruzi*.[69] Anti-*T. cruzi* IgG3 increased 70 days after infection with TcII compared with the other groups.

The heterogeneity of *T. cruzi* infection results in various aspects of immune responses, including inflammatory parameters and the development of tissue damage. The smaller profile of the production of anti-*T. cruzi* antibodies was verified in the QMM5 group and could be correlated with the genetic characteristics of the group. The ELISA technique complemented the biological results and provided information on the TcI and TcII groups, which were the most divergent in molecular terms. The biological and molecular results from these 11 *T. cruzi* strains clarified key factors involved in the biology of the parasite and its hosts. These factors included the classification of the strains into the TcI, TcII, and TcIII groups, the finding that TcI presented the highest rate of macrophage infection, and the higher growth kinetic values in the TcII group. These findings underscore the complexity of the pathogenesis of Chagas disease and the influence of the heterogeneity of *T. cruzi* strains on the pathophysiology of disease. Improving the comprehension of these processes will lead to improvements in drug development and the treatment of the disease.

Received March 11, 2016. Accepted for publication January 29, 2017.

Published online January 8, 2018.

Acknowledgments: We thank Julio César Rente Ferreira Filho and Vagner José Mendonça, who collected *Triatoma lenti*, Eliane Góes Nascimento, from the Health Department of the State of Bahia/SESAB—Entomology Division, who sent us specimens of *Triatoma melanocephala*.

Financial support: Financial support was provided by the Brazilian agency known as the CAPES—Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasília, DF, Brazil, grant no. 23038.005285/2011-2012. Support was also provided by PADC—the Programa de Apoio ao Desenvolvimento Cientifico da Faculdade de Ciências Farmacêuticas do Campus de Araraquara da Unesp. FUNDECIF (Fundação para Desenvolvimento das Ciências Farmacêuticas), Araraquara, São Paulo, Brazil.

Disclaimer: The experiments undertaken comply with the current laws of the country in which they were performed.

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