Molecular and biological characterization of *Trypanosoma cruzi* strains isolated from children from Jequitinhonha Valley, State of Minas Gerais, Brazil

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ABSTRACT

Introduction: The biological diversity of *Trypanosoma cruzi* strains plays an important role in the clinical and epidemiological features of Chagas disease. Methods: Eight *T. cruzi* strains isolated from children living in a Chagas disease vector-controlled area of Jequitinhonha Valley, State of Minas Gerais, Brazil, were genetically and biologically characterized. Results: The characterizations demonstrated that all of the strains belonged to *T. cruzi* II, and showed high infectivity and a variable mean maximum peak of parasitemia. Six strains displayed low parasitemia, and two displayed moderate parasitemia. Later peaks of parasitemia and a predominance of intermediate and large trypomastigotes in all *T. cruzi* strains were observed. The mean pre-patent period was relatively short (4.2±0.25 to 13.7±3.08 days), whereas the patent period ranged from 3.3±1.08 to 34.5±3.52 days. Mortality was observed only in animals infected with strain 806 (62.5%). Histopathological analysis of the heart showed that strains 501 and 806 caused inflammation, but fibrosis was observed only in animals infected with strain 806. Conclusions: The results indicate the presence of an association between the biological behavior in mice and the genetic characteristics of the parasites. The study also confirmed general data from Brazil where *T. cruzi* II lineage is the most prevalent in the domiciliary cycle and generally has low virulence, with some strains capable of inducing inflammatory processes and fibrosis.

Keywords: *Trypanosoma cruzi* strains. Jequitinhonha Valley. Molecular characterization. Biological characterization.

INTRODUCTION

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and is an important tropical disease that affects 10 million people worldwide, mostly in Latin America where it is endemic. It is estimated that over 10,000 people die each year due to the clinical manifestations of Chagas disease, which mainly affects the heart and the gastrointestinal tract. It is well established that *T. cruzi* is a complex taxon that exhibits great genetic variability. *Trypanosoma cruzi* is distributed into six (I to VI) discrete taxonomic units (DTUs) that show significant differences with respect to their ecological and geographic distributions. The clonal nature of *Trypanosoma cruzi* predicts a linkage between its genetic variability and biological properties, such as morphology, virulence, pathogenesis, mortality, and drug susceptibility, as demonstrated under experimental conditions.

Furthermore, geographical variations in the prevalence of the different clinical manifestations of Chagas disease have also been observed, and these variations were associated with the genetic heterogeneity of *T. cruzi* populations. Some authors have demonstrated that different *T. cruzi* strains cause peculiar tissue lesions due to their specific and predominant tropisms in different mammalian cells, such as macrophages, cardiac and skeletal muscle cells, and neurons.

Considering the influence of the genetic characteristics of *T. cruzi* populations on their biology, epidemiology, and therapeutic susceptibility/resistance in *vitro* and in *vivo*, the goal of this study was to characterize the genetics, biology, and morphology of *T. cruzi* samples isolated from children living in a vector-controlled area endemic for Chagas disease in Jequitinhonha Valley, State of Minas Gerais, Brazil. This study may aid further studies concerning the clinical and therapeutic management of patients within the region studied.
Patients and Trypanosoma cruzi strains

Eight children were identified through a serological inquiry of Chagas disease in Berilo and José Gonçalves de Minas, Jequitinhonha Valley, State of Minas Gerais (MG), Brazil, from a total of 1,412 samples analyzed. Five children (8-16 years old) were from Berilo, and three (11-16 years old) were from José Gonçalves de Minas. All of the children presented with the indeterminate clinical form of the disease. These municipalities are very close to each other (12km apart). One T. cruzi strain was isolated from each child, and a total of eight T. cruzi strains were isolated by hemoculture. The strains were identified as 1661, 501, 2405, 817, 795, 829, 806, and 855.

Molecular typing

Epimastigote forms obtained from the liver infusion tryptose (LIT) cultures were washed with phosphate-buffered solution (PBS) by centrifugation at 4°C and 3,500rpm. The parasite pellets were stored at -70°C. For molecular typing, polymorphisms of isoenzyme profiles and the triplice assay that explore the combined analysis of 24Sα ribosomal DNA (rDNA) gene, the mini-exon intergenic regions (ITR) gene, and the cytochrome oxidase subunit II (COII) gene polymorphism of T. cruzi were employed.

Isoenzyme profiles: the parasite pellets were submitted to enzymatic extraction. Six enzymatic systems were used: glucose-6-phosphate isomerase (GPI, E.C. 5.3.1.9), glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.44), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), glutamate dehydrogenase nicotinamide adenine dinucleotide (NAD+) (GDH1, E.C.1.4.1.2), glutamate dehydrogenase nicotinamide adenine dinucleotide phosphate (NADP+) (GDH2, E.C.1.4.1.4), and malic enzyme (ME, E.C.1.1.1.40). The analyses were performed in a horizontal system (Helena, Beaumont, TX, USA) using an acetate-cellulose membrane (Helena, Beaumont, TX, USA). The isoenzyme profile was compared with the reference T. cruzi stocks from different DTUs, including P209 cl1 (U.S.A), the isoenzyme profile was compared with the reference T. cruzi stocks from different DTUs, including P209 cl1 (U.S.A).

Polymorphism of the COII gene: for molecular characterization, parasite DNA was extracted, and the mitochondrial COII gene was amplified using the primers Tcmit-10 (5´-CCATATTTTGCGCTATT-3´) and Tcmit-21 (5´-TTGTAATAGGATCAGTTT-3´) to produce a 375bp fragment from T. cruzi maxicircle DNA. Each PCR was performed in a final volume of 15µL containing 10mM Tris-HCl (pH 8.4), 50mM KCl, 3mM MgCl2, 250µM of each dNTP, 0.3µM of each primer, 0.5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 1µL of T. cruzi DNA covered with mineral oil. The PCR amplification cycles consisted of an initial denaturation of 3min at 94°C, annealing at 68°C for 1min, extension at 72°C for 1min and denaturation at 94°C. Every three cycles, the annealing temperature was decreased by 2 degrees to 66, 64, 62, and 60°C. At the final temperature, the number of cycles was increased to 35 and was followed by a final extension step at 72°C for 10min. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. DNA obtained from strains and clones that yielded approximately 200bp (231, T. cruzi III) and 150bp (CL Brener, T. cruzi VI) fragments were used as reference amplicons for the SL-ITR gene.

Polymorphism of the ITR gene: molecular typing of the ITR spliced leader was performed using the primers TcIII (5´-CTCCCCAGTGGCCTGGG-3´) and UTCC (5´-CTGACCAATATAGACAGAACTG-3´). This PCR strategy targeting the SL-ITR gene was devised to distinguish populations belonging to T. cruzi III and IV (amplicons of approximately 200bp) from populations of T. cruzi I, II, V, and VI, which present fragments of approximately 150bp. The reactions were performed in a total volume of 15µL containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 3mM MgCl2, 250µM of each dNTP, 3µM of each primer, 0.5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 1µL of T. cruzi DNA covered with mineral oil. The PCR amplification cycles consisted of an initial denaturation of 3min at 94°C, annealing at 68°C for 1min, extension at 72°C for 1min and denaturation at 94°C. Every three cycles, the annealing temperature was decreased by 2 degrees to 66, 64, 62, and 60°C. At the final temperature, the number of cycles was increased to 35 and was followed by a final extension step at 72°C for 10min. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. DNA obtained from strains and clones that yielded approximately 200bp (231, T. cruzi III) and 150bp (CL Brener, T. cruzi VI) fragments were used as reference amplicons for the SL-ITR gene.

Biological characterization

Animals and experimental Trypanosoma cruzi infection: Female swiss mice (28-30 days old) obtained from the CCA (Centro de Ciência Animal) of the Universidade Federal de Ouro Preto (UFOP), MG, Brazil, were used in this study.
The study was approved by the Ethics Committee in Animal Experimentation of UFOP, MG, Brazil (Process 2009/10). The animals were maintained according to the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA).

Metacyclic trypomastigotes obtained from LIT cultures were intraperitoneally inoculated into the mice. For each T. cruzi strain, 8 mice were infected with 1.0 x 10^6 blood trypomastigotes from previously infected animals. The following parameters were evaluated:

**Infectivity and mortality:** infectivity (%INF), defined as the percentage of mice that presented a positive fresh blood examination (FBE) and/or hemoculture (Hm); and mortality (%MOR), which was registered daily after inoculation and expressed as the cumulative percentage of death throughout the infection period.

**Fresh blood examination:** five microliters of blood collected from the tail vein were examined on alternate days, and parasitemia was evaluated. The examination began four days after inoculation and continued for five consecutive days after negative examination. The results were expressed as the percentage of mice with positive FBE (%+FBE). The following parameters were also assessed: pre-patent period (PPP), patent period (PP), maximum peak of parasitemia (MPP), and day of maximum peak of parasitemia (DMPP).

**Morphology of blood trypomastigotes:** parasite morphology was evaluated daily by microscopic examination of fresh blood obtained from the mouse tail vein during the acute phase of infection. The percentages of slender, intermediate, and large parasites were recorded.

**Hemoculture:** thirty days after infection, the animals that had negative FBE results were subjected to hemoculture. Each tube of culture was examined for the presence of parasites after 30, 60, 90, and 120 days, and the results were expressed as the percentage of mice with a positive Hm (%+Hm).

**Histopathology:** for each T. cruzi strain, three infected mice were euthanized during the chronic phase of the infection (290 days after inoculation). The heart was fixed in 10% buffered formalin (pH 7.2) and embedded in paraffin. Sections (5µm thick) were mounted on glass slides and stained with hematoxylin-eosin. Morphometric studies of inflammation involved the analysis of 15 randomly selected fields (total area, 1.1 x 10^4µm²) of tissue sections on a single slide per animal. Inflammatory infiltration in the heart was quantified by counting the cellular nuclei. Animals that were not infected showed an average of 200 cellular nuclei in their cardiac muscular tissue. Thus, presence of inflammation was considered when the heart of a mouse presented more than 200 cellular nuclei. Images registered with a 40× objective were analyzed with Leica QWin software (Leica Microsystems, Wetzlar, Germany).

**Statistical analysis**

The data for the biological parameters (PP, PM, DPMP, and area under the parasitemia curve) were analyzed using the program Prism 5 for Windows, version 5.0. The Kolmogorov-Smirnov test of normality was used for data corresponding to all parameters. Data with normal distributions were evaluated by analysis of variance (ANOVA) followed by the Newman-Keuls post-test. For the data that were not normally distributed, the non-parametric Mann-Whitney test was employed. The analyses of mortality and infectivity were performed using the chi-square test of homogeneity. Statistical significance was defined as p < 0.05.

**Ethical considerations**

The inclusion of patients in the study and the blood collections were performed after the patients signed a consent form approved by the Ethics Committee for Research in Humans from René Rachou Research Center (CPqRR), of the Oswaldo Cruz Foundation (FIOCRUZ), Belo Horizonte, MG (Process Number 007/02).

**RESULTS**

**Molecular typing**

The molecular characterization revealed that all of the T. cruzi strains analyzed had genetic profiles indicative of T. cruzi II. Figures 1A and B show the typical electrophoretic profiles for GPI and G6PD, respectively.

Analysis of the 24Sα rDNA gene revealed the presence of the 125bp fragment (rDNA type 1) in all strains, compatible with T. cruzi II or T. cruzi VI (Figure 2A). The analysis of the amplified products of ITS revealed the presence of 150bp fragments that were compatible with T. cruzi II or T. cruzi VI for all strains (Figure 2B). The restriction fragment length polymorphism (RFLP) profiles for the COII gene showed that all of the strains had the 81 and 212bp fragments related to mitochondrial haplotype C that are characteristic of T. cruzi II (Figure 2C).

**Biological characterization**

**Infectivity and mortality:** the percentage of infectivity (%INF) observed in mice inoculated with six T. cruzi strains (795, 806, 817, 829, 1661, and 855) isolated from children was 100%, as demonstrated by a positive FBE and Hm for all of these strains (Table 1). It was necessary to carry out Hm to confirm infection in three animals. One animal infected with strain 829 was positive by Hm, but the animals infected with strains 501 and 2405 were negative (Table 1). The experimental infections with strains 501 and 2405 both had a %INF of 87.5% (Table 1). No significant differences were observed between strains with respect to this parameter.

No mortality was observed in the majority of the animals infected with the eight T. cruzi strains during the acute and chronic phases of infection; the exception was strain 806, which was associated with a mortality rate of 62.5% (5/8) in the first month of infection (Table 1). Regarding this parameter, this strain was significantly different from the other strains studied.

**Parasitemia curve:** to classify the Trypanosoma cruzi strains, an arbitrary criterion was used: low parasitemia (<300,000/0.1mL of blood), moderate parasitemia (300,000-1,000,000/0.1mL of blood), and high parasitemia (>1,000,000/0.1mL of blood). Six strains...
FIGURE 1 - Electrophoretic profiles of the glucose-6-phosphate isomerase (A) and glucose-6-phosphate dehydrogenase (B) enzymes from Trypanosoma cruzi strains isolated from children living in Berilo and José Gonçalves de Minas, Jequitinhonha Valley, State of Minas Gerais, Brazil. Reference stocks: P209 cl1 (T. cruzi I), MAS cl1 (T. cruzi II), CM17 (T. cruzi III), CAN III cl1 (T. cruzi IV), Bug 2148 cl1 (T. cruzi V), and TULA cl2 (T. cruzi VI). Trypanosoma cruzi strains isolated from children: 855, 795, 817, 829, 2405, 1661, 806, and 501.

(829, 795, 855, 817, 501, and 2405) showed low parasitemia, and two strains (806 and 1661) demonstrated moderate parasitemia (Figure 3 and Table 1). Samples 806 and 1661 showed moderate parasitemia and presented higher numbers of parasites in the peripheral blood ($p \leq 0.05$) than the other strains, but no significant differences were observed between them (Figure 3 and Table 1). Significant differences were also detected between the areas under the parasitemia curves of strains 795 and 829. No differences were detected when the other pairs of strains were compared.

Strains 806 and 1661 were more virulent (higher parasitemia) than the other strains and exhibited the lowest mean PPP (6.0 days for strain 806 and 4.2±0.25 days for strain 1,661, Table 1). These strains showed an earlier mean DMPP, which was 15.75±2.15 days for the 806 strain and 17.7±0.79 days for the 1,661 strain (Table 1).

Strain 829 exhibited the lowest number of parasites in the peripheral blood during the PP (5.500 trypomastigotes/0.1mL of blood), a longer PPP (13.7±3.08), and a shorter PP (3.3±1.08) compared with the other strains (Table 1). This strain was also the least virulent (Figure 3).

In general, the mean PPPs were relatively short and oscillated between 4.2±0.25 and 13.7±3.08 days, whereas the PP was between 3.3±1.08 and 34.5±3.52 days. The MPP values oscillated between 5.5±1.45 and 840.9±364.43 x 103 trypomastigotes/0.1mL of blood. A DMPP was also observed, with values between 15.75±2.15 and 26.25±4.86 days. The biological parameters of all of the tested strains of T. cruzi are shown in Table 1.

Morphological analysis of blood trypomastigotes revealed a predominance of intermediate and large forms for all strains. Strain 806 exhibited the highest percentage of slender trypomastigotes, which disappeared as the infection progressed.

Histopathology: histopathological analyses of the hearts of mice infected with the T. cruzi strains showed mononuclear infiltrate cells during the chronic phase of the infection in the animals infected with strains 501 and 806 (Figure 4), and fibrosis was detected only in animals infected with strain 806 (data not shown). Inflammation and fibrosis were absent during the chronic phase of the infection in the animals infected with the other strains.

DISCUSSION

Variations in the clinical forms of Chagas disease and in the susceptibility/resistance of T. cruzi to specific treatment have been observed during evaluations of patients from distinct endemic regions. These differences may be attributed to T. cruzi and host genetic diversity in addition to environmental conditions, which were the same for all experimental groups in this study and were consequently not considered in our analyses.

These observations have prompted several research groups to isolate and characterize different samples of the parasite from distinct hosts and vectors species from domestic, peridomestic, and sylvatic environments. Differences in T. cruzi strains or clones related to biological behavior in vertebrate and invertebrate hosts, resistance to drugs, induction of immune response, and geographical distribution were also demonstrated.
Because parasite genetics and biology may be associated with the clinical aspects of the disease\cite{33,34}, the goal of the present work was to determine the characteristics of \textit{T. cruzi} strains isolated from children in the Jequitinhonha Valley, MG, Brazil, an endemic area where the clinical aspects of Chagas disease have not yet been well characterized. Additionally, the correlation between a parasite's genotype and biology was explored.

The molecular typing revealed that all of the strains studied were \textit{T. cruzi} II, similar to other endemic, central regions of Brazil\cite{16,35-38} and other countries of the South Cone in Latin America\cite{39,40}. Other researchers have studied samples of \textit{T. cruzi}
isolated from patients residing in Virgem da Lapa, a municipality near the studied region\textsuperscript{42}, and they detected the presence of the same genetic group found in our study. However, it is important to note that the author of the Virgem da Lapa\textsuperscript{42} study used an isoenzymatic characterization method that is unable to differentiate \textit{T. cruzi} II from hybrid groups. The biological characterization showed that the majority of the strains of \textit{T. cruzi} isolated from children were infective for Swiss mice, presented low virulence because six of eight strains showed low parasitemia, later peaks of parasitemia, had a predominance of large blood trypomastigotes throughout the acute phase, and showed low mortality rates, characteristics well-matched with \textit{T. cruzi} II\textsuperscript{8,11,12}. For strains 806 and 1661, moderate parasitemia was observed, particularly for strain 806, which displayed a mortality rate of 62.5%. However, during five successive blood passages (data not shown) in animals infected with this same strain, no mortality was observed, which confirms the low virulence characteristic of \textit{T. cruzi} II strains. These results corroborate previous studies that demonstrated that the majority of the \textit{T. cruzi} strains isolated from humans living in different states of central and southern Brazil\textsuperscript{11,43-45}, including Virgem da Lapa\textsuperscript{42} and Berilo\textsuperscript{59} cities of Minas Gerais, presented low virulence and mortality in mice.

The higher parasitemia of strain 806 may be associated with a high percentage of slender blood trypomastigotes early in the infection; this observation is consistent with the results of other studies\textsuperscript{11,28,29,42,46}. The PPP and the DMPP for strains 806 and 1,661, which displayed a mortality rate of 62.5%. However, during five successive blood passages (data not shown) in animals infected with this strain, no mortality was observed, what confirms the low virulence characteristic of \textit{T. cruzi} II strains. These results corroborate previous studies that demonstrated that the majority of the \textit{T. cruzi} strains isolated from children living in different states of central and southern Brazil\textsuperscript{11,43-45}, including Virgem da Lapa\textsuperscript{42} and Berilo\textsuperscript{59} cities of Minas Gerais, presented low virulence and mortality in mice.

As observed in the present study, other authors\textsuperscript{46,47} have demonstrated that strains that multiply slowly present a later peak of parasitemia, are predominantly myotropic, and show a predominance of large blood trypomastigotes during the acute phase of infection. Thus, the present study demonstrated for the first time that \textit{T. cruzi} strains isolated from children living in the Berilo and José Gonçalves de Minas municipalities in Jequitinhonha Valley, MG, which were genetically characterized as \textit{T. cruzi} II with appropriate molecular markers\textsuperscript{16}, showed similar biological characteristics in mice, as in other experimental studies\textsuperscript{7,8,13,49}.

\begin{table}[h]
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\begin{tabular}{cccccccc}
\hline
\textbf{Strains} & \textbf{PPP} & \textbf{PP} & \textbf{MPP x 10\textsuperscript{3}} & \textbf{DMPP} & \textbf{\% +FBE} & \textbf{\% +Hm} & \textbf{\% INF} & \textbf{\% MOR} \\
\hline
829 & 13.7 ± 3.08 & 3.3 ± 1.08 & 5.5 ± 1.45 & 18 ± 4.05 & 87.5 (7/8) & 100.0 (1/1) & 100.0 (8/8) & 0.0 \\
795 & 9 ± 0.65 & 34.5 ± 3.52 & 68.6 ± 17.17 & 21.5 ± 2.44 & 100.0 (8/8) & NP & 100.0 (8/8) & 0.0 \\
855 & 9.7 ± 0.59 & 21.5 ± 3.75 & 98.1 ± 31.70 & 18.2 ± 0.79 & 100.0 (8/8) & NP & 100.0 (8/8) & 0.0 \\
817 & 8 ± 0 & 26.25 ± 3.14 & 238.2 ± 84.61 & 23.5 ± 0.82 & 100.0 (8/8) & NP & 100.0 (8/8) & 0.0 \\
806 & 6 ± 0 & 20.2 ± 1.79 & 840.9 ± 364.43 & 15.75 ± 2.15 & 100.0 (8/8) & NP & 100.0 (8/8) & 62.5 (5/8) \\
1661 & 4.2 ± 0.25 & 27.2 ± 1.19 & 471.7 ± 96.75 & 17.7 ± 0.79 & 100.0 (8/8) & NP & 100.0 (8/8) & 0.0 \\
501 & 9.5 ± 1.45 & 25 ± 4.0 & 122.5 ± 43.94 & 21 ± 3.52 & 87.5 (7/8) & 0.0 (0/1) & 87.5 (7/8) & 0.0 \\
2405 & 13.5 ± 2.02 & 23.25 ± 5.80 & 58 ± 16.71 & 26.25 ± 4.86 & 87.5 (7/8) & 0.0 (0/1) & 87.5 (7/8) & 0.0 \\
\hline
\end{tabular}
\caption{Means of the biological parameters evaluated in Swiss mice inoculated with the \textit{Trypanosoma cruzi} strains isolated from children living in Berilo and José Gonçalves de Minas, Jequitinhonha Valley, State of Minas Gerais, Brazil.}
\end{table}
Finally, the results obtained in this study reinforce the idea that both the genetic and biological characterization of *T. cruzi* strains may be important because these methods are the most appropriate to determine whether there is a correlation between these characteristics and the clinical-pathological manifestations of Chagas disease, a polemic and complex theme in Chagas disease. Therefore, we estimate that this work may contribute to a better understanding of the state of Chagas disease in the Jequitinhonha Valley. This knowledge may help our team provide clinical assistance and etiological treatment for the patients in the studied region, which is considered one of the most important endemic areas of this disease in our country.

**ACKNOWLEDGMENTS**

We are thankful to the municipal authorities and the local health teams in Berilo and José Gonçalves de Minas for their support in the development of this study.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**FINANCIAL SUPPORT**

We would like to thank Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) (Rede Mineria de Bioterismo/FAPEMIG), announcement Programa de Pesquisa para o Sistema Único de Saúde (PPSUS/05), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Agência Brasileira da Inovação-Fundo de Infra-Estrutura (FINEP-CTInfra), Secretaria de Educação Superior/Ministério da Educação (SESU/MEC), and Pró-Reitoria de Extensão/Universidade Federal de Ouro Preto (PROEX/UFOP) for providing financial support for this study and the author’s research fellowship.

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