Persistence of PCR-positive tissue in benznidazole-treated mice with negative blood parasitological and serological tests in dual infections with *Trypanosoma cruzi* stocks from different genotypes

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Objectives: To assess different methodologies to better define an early post-therapeutic cure criterion after benznidazole treatment in BALB/c mice following mixed infection with dual *Trypanosoma cruzi* genotypes.

Methods: According to the classical cure criteria, animals were classified as treated not cured (TNC = 76.4%), treated cured (TC = 12.5%) and dissociated (DIS = 11.1%) using parasitological [fresh blood examination (FBE), blood culture (BC) and blood PCR] and serological methods [conventional serology (CS-ELISA) and non-conventional serology (NCS-FC-ALTA)]. Tissues were also evaluated by PCR. Results: FBE was able to detect patent parasitaemia in only 18.1% of TNC and therapeutic failure was detected in 79.1% and 97.2% of TNC by BC and blood PCR, respectively. CS-ELISA should not be used before 3 months after treatment since it may lead to false-negative results. At 3 months after treatment with benznidazole, NCS-FC-ALTA was more efficient for categorizing the groups of treated mice. In the TNC group, although a decreased frequency of PCR-positive tissue was observed in several host tissues, increased positivity was also observed, despite the *T. cruzi* genotype combination. All TC animals presented at least two positive tissue-PCR results.

Conclusions: Our results confirm that NSC-FC-ALTA and blood PCR are the most suitable methods to early detect therapeutic failure in acute murine *T. cruzi* infection. Additionally, our data show that BC positivity is highly dependent upon the *T. cruzi* genotype combination. Moreover, our findings demonstrated that PCR tests performed on tissues from animals considered cured after benznidazole treatment still detected *T. cruzi* DNA, most probably indicating residual infection.

Keywords: mixed infection, genetic diversity, cure control, murine model

Introduction

It is estimated that 13 million individuals still remain infected by *Trypanosoma cruzi* in Latin America1 and ~30% of the infected individuals develop cardiac or digestive forms of chronic disease.2 Therefore, development of new drugs for this disease is a priority.3 In this context, experimental studies in a murine model are used to test dosing regimens in the search for effective
Materials and methods

Parasites

Eight T. cruzi stocks representative of four major genotypes \(^{23}\) were used: 19 (Gamba cl1 and OPS21 cl1) and 20 (Cuica cl1 and P209 cl1) both from T. cruzi \(^{15}\), genotype 32 (IVV cl14 and MAS cl11) from T. cruzi II and the hybrid genotype 39 (Bug2148 cl1 and SO3 cl15).

Experimental design and infection

Groups of twelve 28–30-day-old BALB/c female mice from Instituto de Ciências Biológicas, UFMG, Brazil, were intraperitoneally inoculated with 5000 blood trypanosomes of each T. cruzi stock in dual infections. The number of parasites was determined according to Brener. \(^ {7} \) Twenty-four different parasite combinations were evaluated in dual infections. The study was approved by the Ethics Committee in Animal Experimentation of Federal University of Ouro Preto (UFOP), MG, Brazil, Process number 2007/77. Animals were maintained according to the guidelines of the Brazilian School of Animal Experimentation (COBEA).

Treatment schedule

Treatment was performed during the acute phase of infection, starting at day 10 following inoculation, including only those animals where infection was confirmed by fresh blood examination (FBE) or blood culture. Groups of six animals were treated with benznidazole (Rochagan \(^{8}\), Roche) at 100 mg/kg body weight for 20 consecutive days. The drug was suspended in gum arabic and administered by gavage. Groups of six infected and untreated animals as well as non-infected controls were always included in each experimental batch.

Cure assessment

The analysis of therapeutic efficacy following treatment with benznidazole was defined by cure criteria based on both serological [conventional serology (CS-ELISA) and non-conventional serology (NCS-FC-ALTA)] and parasitological methods [FBE, blood culture (BC) and blood PCR], following the full cure criteria as described by Krettli and Brener. \(^ {16} \) Taking together the results from the serological and parasitological tests used in this study to assess the overall cure criteria, the animals treated with benznidazole were grouped as follows: treated not cured (TNC), animals presenting at least one positive parasitological test and positive CS-ELISA and NCS-FC-ALTA (FBE/BC/blood PCR); and dissociated (DIS) animals presenting negative results in all parasitological tests (FBE/BC/blood PCR) and NCS-FC-ALTA, but positive CS-ELISA; and treated cured (TC) animals presenting negative results in all parasitological and serological tests.

Parasitological methods

**Fresh blood examination.** Blood samples from the tail vein were collected daily and exhaustively examined by optical light microscopy for detection of living trypanosomes until 30 days after treatment.

**Blood culture.** The cultures were carried out 30 days after treatment according to Filardi and Brener. \(^ {15} \) Blood samples collected from the orbital sinus vein were inoculated into 3 mL of liver infusion tryptose (LIT) medium and maintained at 28°C. Each tube was examined for detection of parasites at days 30, 60, 90 and 120 following culture.

**PCR on peripheral blood.** Blood samples were collected from the orbital sinus vein at day 30 after treatment, mixed in a 1:2 proportion with 6 M guanidine/0.2 M EDTA, pH 8.0, and stored at room temperature. \(^ {30} \) DNA extraction was carried out according to a modified Gomes et al. \(^ {31} \) method. PCR amplifications were carried out in 9 μL of reaction mixture containing Tris–HCl 10 mM (pH 9.0), 0.1% Triton X-100, 75 mM KCl, 3.5 mM MgCl\(_2\), 0.2 mM of each deoxynucleotide (Sigma), 0.5 U of Platinum Taq DNA polymerase (Invitrogen) and 10 pmol of each oligonucleotide primer (S35 and S36 described by Avila et al. \(^ {36} \) and provided by Invitrogen). Two microflites of the blood DNA sample was added to the reaction mixture, which was then overlaid with 30 μL mineral oil to avoid evaporation. After an initial denaturation step of 5 min at 94°C, 35 cycles of amplification were performed in a Thermal cycler (MJ Research, PTC-150), each one consisting of 1 min at 95°C for DNA denaturation, 1 min at 65°C for primer annealing and 1 min at 72°C for primer extension followed by a final extension.
step of 10 min at 72°C. Amplified DNA was visualized in silver-stained 6% polyacrylamide gels. Positive, negative and reagent controls were processed in parallel in each assay.

Serological methods

Conventional serology. Enzyme-linked immunosorbent assays were performed according to a modification of the method of Voller et al. Samples of mouse serum were collected 3 months after treatment and stored at −20°C. Sera were tested at 1:80 dilution in PBS using T. cruzi antigen prepared by alkaline extraction of Y strain parasites obtained at exponential growth in LIT medium. Antibody binding was detected by using peroxidase-labelled anti-mouse immunoglobulin G (Sigma Immunochemical Reagents, St Louis, MO, USA). The absorbance (A) was read in a spectrophotometer with a 490 nm filter (model 3550, Bio-Rad). Positive and negative controls were processed in parallel in each assay. The cut-off value was calculated for each plate considering the mean absorbance of 10 negative control serum samples plus two standard deviations.

To evaluate the performance of individual CS-ELISA to be used in the cure criteria, blood serum samples were initially collected at 2 and 3 months after treatment from TNC* (positive NCS FC-ALTA and FBE/BC/blood PCR) and TC* (negative NCS-FC-ALTA and FBE/BC/blood PCR) and tested by CS-ELISA to determine the minimum time after treatment required to guarantee the correct classification of treated animals following the full cure criteria described by Krelli and Brenner. Non-conventional serology. The flow cytometry technique, to detect anti-live trypomastigote antibodies (FC-ALTA), was carried out in serum collected 3 months after treatment as described by Martins-Filho et al. The serum from experimental animals was assayed at 1:1500 dilution using goat anti-mouse IgG (Sigma Immunochemical Reagents) labelled with fluorescein isothiocyanate (FITC) to assay the IgG reactivity as described by Toledo et al. The results were expressed as the percentage of positive fluorescent parasites (PPFP) based on the internal control of non-specific binding of the FITC-conjugated second-step reagent. Positive and negative controls were included in all experimental batches. Flow cytometric measurements were performed on FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA). Samples were considered negative when PPFP was \( \leq 20\% \) and positive when PPFP was >20% as described by Martins-Filho et al.

Tissue-PCR analysis

The principal tissues of mice were evaluated to assess whether there is correlation between the results of the conventional cure criterion and tissue-PCR.

PCR was carried out on tissues of two randomly selected animals from each mixed infection; a total of eight mice for each genotype combination. Animals inoculated with the mixture 20 + 19 were not cured, and for this reason, they were not examined by this technique. The same occurred with animals of the mixture 20 + 32 because when treated and cured animals (TC) were selected, unfortunately, the cured mice were not included. Animals were necropsied 3 months after treatment (120 days after infection). Heart, skeletal muscle, colon, spleen, bladder and brain specimens were collected, exhaustively washed with isotonic saline, divided into 5 mm² fragments and stored at −70°C. DNA extraction was performed as described by Andrade et al. Thawed tissue samples were completely minced with a surgical scalpel and transferred into microcentrifuge tubes and DNA extracted by alkaline lysis with 20 µL of 50 mM NaOH, followed by neutralization with 100 µL of 130 mM Tris–HCl, pH 7.0. Tissue lysates were 10-fold diluted in sterile water and stored at −20°C. Parasite detection in each tissue lyse was performed using the specific PCR amplification of an ~330 bp fragment, corresponding to the variable regions of the T. cruzi L DNA minicircle. PCR amplifications were carried under the same conditions described for blood DNA. Positive, negative and reagents controls were processed in parallel for each step of tissue-PCR assay.

Statistical analysis

The one-way analysis of variance (ANOVA) was used followed by Newman–Keuls test to compare the CS-ELISA (A\(_{900}\)) and NCS-FC-ALTA (PPFP) results between groups CNT, TNC, DIS and TC. The percentages of positivity obtained for each group using several methods were compared by the Fisher test. The correlation between methodologies was verified using \( \kappa \) coefficient.

Results

Conventional cure criterion

Following the interpretation of cure criteria, animals were classified based on their response to benznidazole treatment, as established for the classic cure criterion methodologies, leading to 76.4% TNC (n = 110), 12.5% TC (n = 18) and 11.1% DIS (n = 16) (Table 1). According to Krelli and Brenner, the total cure rate reached 23.6% (n = 34) by including TC and DIS animals into the group of cured animals. The genotype combination 20 + 19 did not present any DIS or TC animals, whereas the genotype combination 39 + 32 showed higher frequency of DIS animals and the highest TC frequency among the mixtures (Table 1).

To evaluate the performance of individual parasitological methods to be used in the cure criteria, blood samples from 288 animals classified as infected not treated (INT), TNC and TC were tested in parallel using FBE, BC and blood PCR.

In the INT group, the BC and blood PCR were positive in 88.8% and 95.8%, respectively. Despite this lower overall sensitivity of BC for the identification of INT in comparison with

<table>
<thead>
<tr>
<th>Genotype combinations (#)</th>
<th>Groups % (n)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TNC</td>
</tr>
<tr>
<td>20 + 19 (24)</td>
<td>100.0 (24)</td>
</tr>
<tr>
<td>20 + 39 (24)</td>
<td>75.0 (18)</td>
</tr>
<tr>
<td>20 + 32 (24)</td>
<td>87.5 (8)</td>
</tr>
<tr>
<td>19 + 39 (28)</td>
<td>75.0 (21)</td>
</tr>
<tr>
<td>19 + 32 (22)</td>
<td>70.6 (17)</td>
</tr>
<tr>
<td>39 + 32 (22)</td>
<td>40.9 (9)</td>
</tr>
<tr>
<td>Total (144)</td>
<td>76.4 (110)</td>
</tr>
</tbody>
</table>

TNC, treated and not cured animals; DIS, dissociated animals; TC, treated and cured animals. # Number of animals in each group. *Data are expressed as percentage (%) and number of observation (n) with positive results. Different letters represent significant differences among percentage of animals classified as TNC, DIS or TC comparing the different genotypes combinations at \( P < 0.05 \).
blood PCR, all INT animals presented at least one positive parasitological test. In the INT group, all samples with negative BC displayed positive blood PCR. Two out of 128 samples with positive BC displayed negative blood PCR. Although the performance of BC was similar in all genotype combinations, animals infected with the genotype combinations 19 + 39 and 39 + 32 presented a lower frequency of positive results with this parameter when compared with the other genotype combinations. In a panoramic overview, the positivity of FBE, BC and blood PCR was 18.1%, 79.1% and 97.2% in TNC group, respectively (Table 2). Twenty-two out of 23 TNC animals (95.6%) with negative BC displayed positive blood PCR, whereas 2 out of 87 animals with positive BC showed negative PCR. Considering the positivity of each parasitological test, FBE presented the lowest sensitivity whereas BC displayed higher performance in the majority of the genotype combinations, except for 19 + 32 and 39 + 32 (Table 2). In TNC, the blood PCR was the most sensitive method with high positivity in all genotype combinations. Data analysis demonstrated that CS-ELISA performed at 2 months after treatment gave negative results in 8 out of 110 samples from TNC* not compatible with their positive NCS-FC-ALTA and parasitological tests. On the other hand, only 1 out of 115 sera samples from TNC* showed negative CS-ELISA when collected 3 months after benznidazole treatment, leading to a profile compatible with their clinical status defined by the other methodologies, consistent with the therapeutic failure observed in TNC*. Similarly, the analysis of TC* samples also confirmed that serum samples collected at 3 months after treatment showed the most reliable results to confirm the clinical status of these animals (Figure 1, top graph). Our data allowed us to establish that 3 months represented the minimum time required to apply CS-ELISA in the cure criteria applied in murine acute-phase model of T. cruzi dual-clonal infections. Data were generated from two separate experiment batches.

Serum samples collected from 144 dual T. cruzi genotype-infected animals following treatment with benznidazole were submitted in parallel to CS-ELISA and NCS-FC-ALTA. Sera samples from INT (n = 49) and NI (n = 20) mice were used as control. Serological reactivity of treated animals showed three distinct profiles consistent with the serological behaviour of TNC, DIS and TC. The analysis of the serological profiles further emphasizes the ability of NCS-FC-ALTA to efficiently identify the DIS group, which presented positive CS-ELISA likely TNC mice but negative NCS-FC-ALTA similar to TC (Figure 1, middle graphs). Additional analysis of the TNC group, categorized into BC-negative and BC-positive further showed distinct serological profiles in both CS-ELISA and NCS-FC-ALTA, with TNC BC-negative animals displaying lower anti-T. cruzi antibody reactivity (Figure 1, bottom graphs).

The serological tests (CS-ELISA and NCS-FC-ALTA) were 100% reactive in INT group. Only one TNC animal with positive BC was negative by CS-ELISA and borderline in NCS-FC-ALTA (Figure 1). Significant differences were observed between the serological tests and BC (P < 0.001) as well as blood PCR and BC (P = 0.02). Significant differences were observed among the serological tests and BC (P < 0.001) as well as between blood PCR and BC (P = 0.02) in identification of INT and TNC. The highest correlation index observed was between blood PCR and NCS-FC-ALTA (r = 0.875).

### Tissue-PCR analysis

In order to further characterize the usefulness of PCR to assess post-therapeutic cure in T. cruzi infection, we also evaluated the ability of PCR results to detect T. cruzi kDNA in tissue samples following treatment with benznidazole.

#### Tissue-PCR in INT, TNC and TC

Our data demonstrated that tissue-PCR was able to detect T. cruzi kDNA in all tissues of all mixed infections from INT and TNC groups. Although the heart and skeletal muscle have shown higher frequency of positive results in INT and TNC, the bladder also represents an important target tissue to be evaluated in TNC animals. The brain presented fewer positive results in most infected animals, in both INT and TNC (Figure 2, top and middle graphs).

It was interesting to note that 3 months after treatment, all TC animals with negative results in all parasitological (FBE, BC

### Table 2. Percentage of positive parasitological tests considering different T. cruzi genotype combinations

<table>
<thead>
<tr>
<th>Genotypes combinations</th>
<th>INT % (n)*</th>
<th>Blood PCR</th>
<th>TNC % (n)*</th>
<th>Blood PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FBE</td>
<td>BC</td>
<td></td>
<td>FBE</td>
</tr>
<tr>
<td>20 + 19</td>
<td>100.0% (24)</td>
<td>100.0% (24)</td>
<td>95.8% (23)</td>
<td>16.7% (5)</td>
</tr>
<tr>
<td>20 + 39</td>
<td>100.0% (24)</td>
<td>100.0% (24)</td>
<td>100.0% (24)</td>
<td>11.1% (2)</td>
</tr>
<tr>
<td>20 + 32</td>
<td>100.0% (24)</td>
<td>100.0% (24)</td>
<td>100.0% (24)</td>
<td>33.3% (7)</td>
</tr>
<tr>
<td>19 + 39</td>
<td>28.0% (28)</td>
<td>78.0% (22)</td>
<td>96.4% (27)</td>
<td>23.9% (5)</td>
</tr>
<tr>
<td>19 + 32</td>
<td>22.0% (22)</td>
<td>81.8% (18)</td>
<td>100.0% (22)</td>
<td>5.9% (1)</td>
</tr>
<tr>
<td>39 + 32</td>
<td>22.0% (22)</td>
<td>72.7% (16)</td>
<td>81.8% (18)</td>
<td>0.0% (0)</td>
</tr>
<tr>
<td>Total</td>
<td>100.0% (144)</td>
<td>88.8% (128)</td>
<td>95.8% (138)</td>
<td>18.1% (20)</td>
</tr>
</tbody>
</table>

TNC, treated and not cured animals; INT, infected and not treated animals; FBE, fresh blood examination; BC, blood culture; PCR, polymerase chain reaction; #, Number of animals in each group. *Data are expressed as percentage (%) and number of observation (n) with positive results. Significant differences within the same genotype combination are represented by ¥ and †, in comparison to BC and blood PCR at P < 0.05, respectively. Different letters represent significant differences among the percentage of positive BC test comparing different genotypes combinations at P < 0.05. No significant differences were observed between FBE or blood PCR among distinct genotype combinations. ‡FBE was positive in all animals into INT after exhaustive examination and when performed repeatedly, except when genotype 20 was present in the mixture due to its higher parasitaemia.
PCR-positive tissue in mice classically cured of *T. cruzi*

![Graph](https://academic.oup.com/jac/article-abstract/61/6/1319/739975)

**Figure 1.** Serological profile of BALB/c mice infected with *T. cruzi* genotype combinations classified as infected and not treated (INT), not infected (NI), treated and not cured (TNC), dissociated (DIS) and treated and cured (TC), based on the cure criterion proposed by Krettli and Brener. Parallel CS-ELISA analysis was performed at 2 (open symbols) and 3 months (filled symbols) after the end of treatment with benznidazole in sera samples from TNC* and TC* in order to determine the best time to apply the serological cure criterion—top graph. CS-ELISA and NCS-FC-ALTA were further applied in sera samples from INT, NI, TNC, DIS and TC—middle graphs. CS-ELISA and NCS-FC-ALTA were further characterized in TNC BC+ (with blood culture positive) and TNC BC− (with blood culture negative)—bottom graphs. Results are expressed as *A*490 for CS-ELISA and percentage of positive fluorescent parasites (PPFP) for NCS-FC-ALTA. The horizontal dashed line indicates the respective cut-off value used to discriminate negative and positive results.

and blood PCR) and serological tests presented at least two positive tissue-PCR results. Again, the bladder appears together with the heart and the skeletal muscle with higher frequency of positive results (Figure 2, bottom graph). Figure 2 also illustrates a silver-stained 6% polyacrylamide gel showing the *T. cruzi* kDNA amplified by tissue-PCR from a range of samples obtained from two TC animals.

**Tissue-PCR in different genotype combinations.** The influence of *T. cruzi* genotype combinations in the frequency of positive
tissue-PCR was also evaluated. Even considering that this technique was not performed in the groups of mice infected with strains 20 + 19 and 20 + 32 for reasons described in the Materials and methods section, it was verified that when the TC groups of the other mixtures were evaluated, the percentage of positive results was higher in mice infected with mixtures 20 + 39 and 19 + 39 and lower in animals infected with mixtures 19 + 32 and 39 + 32 (Figure 3).

When tissue-PCR was performed on INT groups, it was positive in a higher percentage of mice infected with the mixture 20 + 39, a lower percentage for the mixture 39 + 32 and quite similar results were observed in animals infected with the other genotypes. For the TNC group, the highest percentage of positive results was observed in mice infected with the mixture 19 + 39 and lowest in mixture 19 + 32. In animals infected with the other genotype combinations, the percentages of positive results were intermediate (Figure 3).

When the results of tissue-PCR were analysed considering only the TC groups and each tissue studied separately, it was verified that all tissues were positive in mice infected with the mixtures 20 + 39 and 19 + 39, all samples being positive only in animals infected with the mixture 20 + 39 (Figure 4). The opposite was observed in mice infected with the mixture 19 + 32 where only the heart and the bladder were positive. In animals infected with the mixture 39 + 32, all tissues except the brain were PCR-positive, but the percentages of positivity were lower than those observed in the great majority of tissues of mice infected with the other mixtures.

Discussion

The efficacy of treatment in Chagas’ disease is very hard to demonstrate due to the difficulties in establishing the cure criterion and the limitations of the serological and parasitological methods available to detect the post-therapeutic cure in murine T. cruzi infection. Therefore, a combination of different methods was used here to assess the cure and compared with the results obtained by tissue-PCR, not routinely used for this purpose, in mice dual infected with T. cruzi major genotypes.

Exhaustively repeated FBE was able to detect patent parasitaemia in only 18.1% of TNC animals. These findings are in agreement with Brener6 who demonstrated the limitation of FBE for detection of the parasite in TNC.
Therapeutic failure was detected in 79.1% of the treated animals by using BC. The apparent lack of sensitivity observed for BC was mainly associated with the genotype combinations 39 + 32 and 19 + 32. These findings suggest that BC should not be used alone for cure testing in the acute phase, even in the murine model, since its sensitivity is dependent on the T. cruzi stock involved in the infection as verified before by Toledo et al. and Martins et al. working with the same T. cruzi stocks.

It was interesting to observe that 97.2% therapeutic failure was detected by blood PCR. It is also important to mention that the majority of the TNC animals with negative BC displayed positive blood PCR, which demonstrates the power of this methodology for early determination of therapeutic failure in murine T. cruzi infection. These results corroborate data from other studies that demonstrated the efficiency of blood PCR for both diagnosis and cure control of T. cruzi infection, independently of the parasite genetic group. On the other hand, Camandaroba et al. did not find similar performance of blood PCR applied as cure criterion for T. cruzi infection, with only 37% of the samples from treated animals showing positive blood PCR, despite the positivity of other parasitological methods, such as subinoculation and/or BC. These discrepancies may probably be attributed to differences of the experimental conditions used during the blood PCR procedure.

Our data demonstrated that the conventional serology (CS-ELISA) performed at 2 months after benznidazole treatment led to false-negative results in animals with positive parasitological tests. Serological tests performed 3 months after treatment demonstrated that only one of these animals still had negative CS-ELISA, suggesting that some animals that showed negative results at 2 months were indeed not cured and that CS-ELISA may not be used as an early cure criterion. These serological findings agreed with previous studies that demonstrated in mice the persistence of positive conventional serology for several months after treatment of T. cruzi infection. On the other hand, our results demonstrated that NCS-FC-ALTA was more efficient for the categorization of the different groups of mice regarding their therapeutic response as early as 3 months after the end of benznidazole treatment. It is interesting to note that TNC (BC−) always displayed lower mean reactivity in comparison to TNC (BC+). These results confirmed the general idea that higher parasitaemia and consequently more antigenic stimulation result in higher antibody production as demonstrated by several authors in different vertebrate hosts during chronic T. cruzi infections.

In general, the results of the different methodologies here employed as cure control agree with the results of other authors working with mice, other experimental models and humans. However, the evaluation of therapeutic efficacy by these methods in mixed experimental T. cruzi infection taking into account the genetic diversity of the parasite has not been studied previously.

The results obtained for INT and TNC groups confirmed the higher capacity of NSC-FC-ALTA and blood PCR for the detection of T. cruzi infection in the murine model. Indeed, in the present study, the FC-ALTA and blood PCR were the techniques that presented the best correlation of results and were most appropriate to define early the therapeutic efficacy. Therefore, the combination of one of these techniques with BC may constitute the most suitable approach for the cure assessment in the murine model of T. cruzi infection.

When tissue-PCR analysis of the animals was also performed, positive results were detected in all tissues of all mixed infections of INT and TNC groups, which demonstrates the efficacy of this technique for the detection of parasite kDNA principally in the heart and skeletal muscle, which are known to be common sites for T. cruzi. Additionally, the bladder was an important target of the parasite in TNC group. These results agree with our previous results in single infections with this same set of T. cruzi stocks.

When tissue-PCR analysis of INT and TNC was considered taking into account the genotype combinations, the results were heterogeneous. In general, animals infected with the mixture 39 + 32 were less positive. These data corroborate other parasitological data that reinforce the low virulence of these stocks in single and mixed infections in mice. We also observed that when genotype 39 was present in the mixed infections (except in the mixture...
the highest percentages of tissue-PCR were observed, which agree with previous data from Toledo et al.\textsuperscript{33,34} who verified the preferential presence of this genotype in tissue rather than in the blood.

Interestingly, our data have demonstrated that all 12 animals considered cured by the classical serological and parasitological cure criteria (CS-ELISA, NCS-FC-ALTA, FBE, BC and blood PCR) showed positive results in the tissue-PCR. As observed in TNC group, the heart, muscle and bladder were again the tissues most frequently positive in tissue-PCR. The mixed infections with the highest percentage of positive results included genotype 39. Curiously, in some genotype combinations of TNC (20 + 39, 20 + 32 and 19 + 39) and TC groups (20 + 39, 19 + 39 and 19 + 32), the positivity of tissue-PCR was higher than that observed in the INT group. If the tissue-PCR positivity is accepted as an indication of active infection, we have to bear in mind that in some cases and depending on the parasite genetics, some treatments may increase tissue parasitism as previously demonstrated by us for genotype 39,\textsuperscript{24}

One of the explanations for the unexpected results of positive tissue-PCR of TC group could be the presence of residual DNA even after the elimination of the parasite as described for parasite antigens in the spleen dendritic cells\textsuperscript{6} or the integration of \textit{T. cruzi} DNA in the cell genome.\textsuperscript{44,45} Similar results were also observed by Michailowsky \textit{et al.}\textsuperscript{8}

It is important to note that the capacity of any given drug to eradicate an infection is dependent on intrinsic host features, such as immune status, in addition to the susceptibility of a particular \textit{T. cruzi} genotype to the drug, as well as the drug properties and toxicity in the host and the length of treatment. Indeed, the results of single, relatively short treatments are not sufficient to claim that parasitological cure cannot be achieved in experimental \textit{T. cruzi} infection models. Therefore, the inconsistency of the serological tests and blood parasitological analysis with tissue-PCR results (which measure the true parasite burden in Chagas disease) may simply reflect the obvious fact that the former tests display intrinsically lower sensitivity. Other authors have reported that prolonged treatments with high doses of conventional drugs can lead to complete reversal of inflammatory processes and even fibrosis of \textit{T. cruzi}-chronically infected tissues, strongly suggesting the eradication of the aetiological agent.\textsuperscript{6} It has also been shown that novel drugs have significantly higher anti-\textit{T. cruzi} activity and lower toxicity than benznidazole, against both acute and chronic infections.\textsuperscript{11,16} Furthermore, the need for further investigation is clear to rigorously prove the residual infection in those animals displaying positive tissue-PCR, despite their negative blood parasitological and serological tests.

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transparency declarations

None to declare.

References


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