Method Article

Benznidazole, itraconazole and their combination in the treatment of acute experimental Chagas disease in dogs

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A B S T R A C T

Chagas disease (CD) is a serious public health problem in Latin America and its treatment remains neglected. Benznidazole (BZ) available in Brazil, presents serious side effects and low therapeutic efficacy at chronic phase. This study evaluated the therapeutic efficacy of BZ, itraconazole (ITZ) and BZ + ITZ in dogs infected with VL-10 T. cruzi strain in the acute phase (Ethic protocol number 2013/28). Twenty young mongrel dogs were inoculated with 2.0×10^3 blood trypomastigotes/kg and divided into four groups: treated with BZ, ITZ and BZ + ITZ for 60 days, and control group (INT-infected not treated). The parasitemia of the BZ + ITZ and BZ groups were similar and showed significant reduction compared to the INT group. The ITZ group also showed significant parasitemia reduction compared to the INT group. For cure control the global analysis of hemoculture (HC), blood PCR, conventional serology (CS-ELISA), heart qPCR and histopathology revealed that BZ + ITZ lead to more reduction of parasitemia during the acute phase and heart qPCR positivity, less cardiac damage and total survival than BZ or ITZ. Moreover, two other dogs, one treated with ITZ and other treated with BZ + ITZ, were always negative in all parasitological tests what indicates parasitological cure or that these dogs are in process of cure.

- BZ + ITZ lead to more reduction of parasitemia, total survival, less heart qPCR positivity and cardiac damage.
- According to the classic cure criterion cure was observed only in one dog submitted to BZ + ITZ treatment.
- Two dogs, one treated with ITZ and other treated with BZ + ITZ were always parasitologically negative.

Specification Table

Subject Area:	Biological Sciences
More specific subject area:	Parasitology, Chagas disease chemotherapy
Method name:	NA
Name and reference of original method:	NA
Resource availability:	NA

Method details

Method name

Parasitemia curve (PAR) and fresh blood examination (FBE)

The parasitemia curve (PAR) was evaluated daily during the acute phase of the infection by fresh blood examination (FBE) of five microliters collected from the ear marginal vein. The parasite count was performed according to Brener (1962) [1], adapted from Pizzi (1949) [2] in dogs inoculated with the VL-10 strain started on the 6th day after infection up to consistent negativation for at least five consecutive days or until the death of the animal. For parasites count an optical microscope (Nikon E200) was used. The number of parasites observed in 50 fields (10 in each coverslip 22 x 22 mm corner and 10 fields in the center) was multiplied by the microscope factor calculated [1] as described: count the total of fields of the coverslip in the horizontal and vertical directions, multiply the horizontal number of fields by the vertical one in order to have the total of fields of the coverslip, divide this number obtained by 50 (total of fields in which the parasites are counted) for have the microscope factor. Otherwise, with the use of a micrometric coverslip, the area of the microscope field is obtained ($A = \pi r2$), and the microscope factor may be calculated following the same rationality.

The PAR curve was plotted using the daily mean count of the number of parasites/0.1 ml of blood from the animals inoculated. This evaluation gives an indication of the treatment activity in terms of the PAR showing partial or complete suppression during treatment in the acute phase, which may be confirmed with other parasitological methods of higher sensitivity such as hemoculture and PCR performed in parallel, according to the classic cure criterion (3), plus alternative methodologies such as qPCR of high sensibility and specificity, and histopathological evaluations (inflammation and fibrosis) at a later stage. Fig. 1 shows the curve of parasitemia in parasites known to be partially sensitive (A) and resistant (B) to treatment.

Hemoculture (HC)

The HC technique is illustrated at Fig. 2 and was performed following the methodology described by Chiari et al. (1989) [4], with some modifications. Briefly, (Fig. 2) 10 mL of blood were collected with heparin in a sterile plastic tube from the dog cephalic vein, immediately centrifuged at 1500 xg for 10 min at 4 °C and the plasma separated and discarded. Thereafter, blood cells were rinsed with LIT
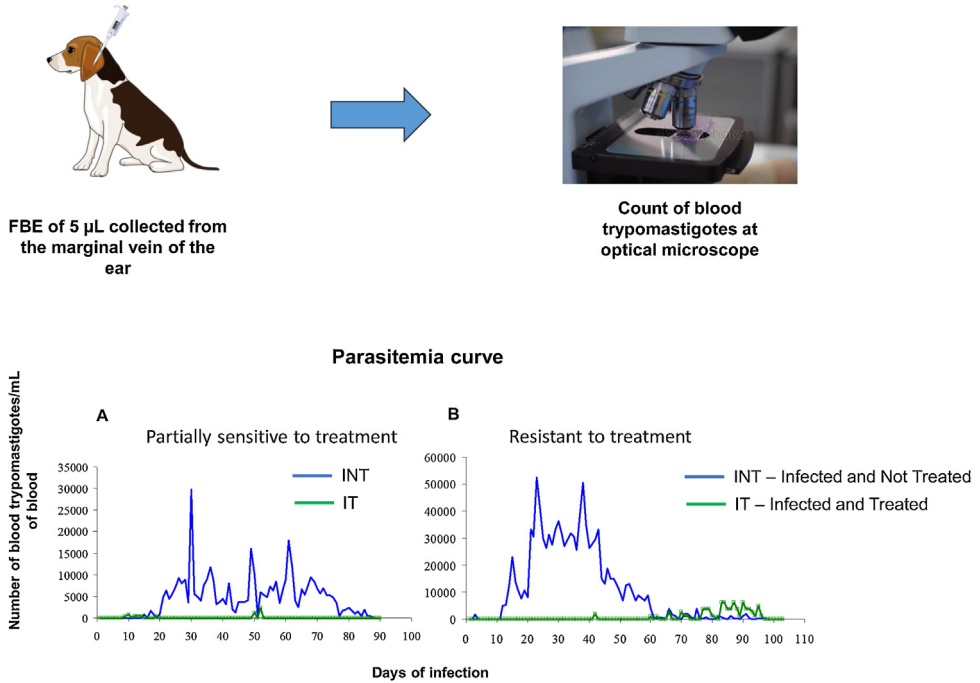
FBE of 5 µL collected from the marginal vein of the ear

Count of blood trypomastigotes at optical microscope

Parasitemia curve

A Partially sensitive to treatment

B Resistant to treatment

Fig. 1. Fresch blood examination (FBE) and Parasitemia curve (PAR).

Leucocytes → Plasma → Hematias

→ LIT → Leucocytes → Blood cells

Leucocytes + LIT

Hematias + LIT

10 mL of blood collected of the cephalic vein

Centrifugation, 3000 rpm, 10 min at 4°C. (plasma discarded)

Blood cells rinsed with LIT medium by centrifugation as previously (LIT discarded)

Incubation in BOD chamber, 28°C and homogenized every 48h

Epimastigotes and tripomasitigotes

Drop of culture at optical microscope 30, 60, 90 and 120 days after culture
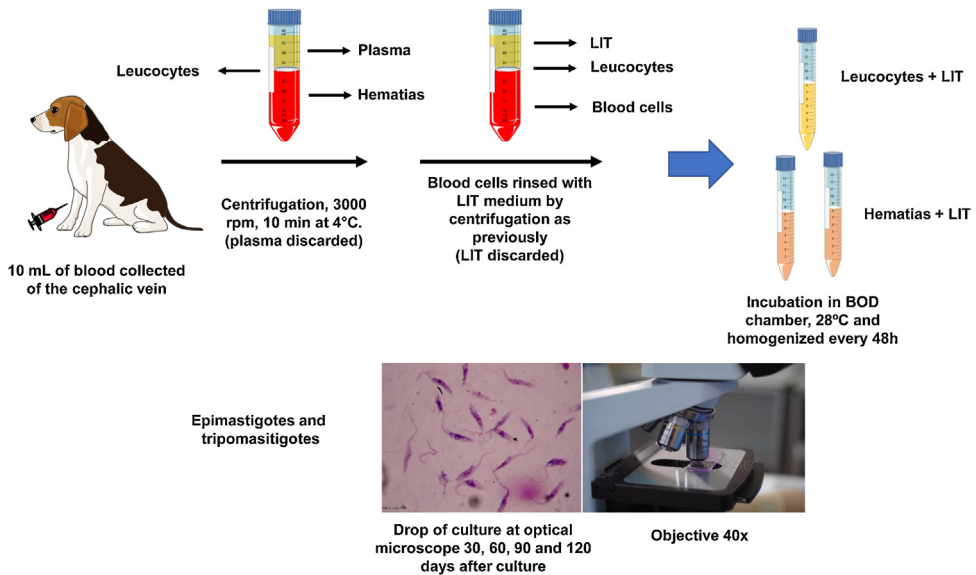
Objective 40x
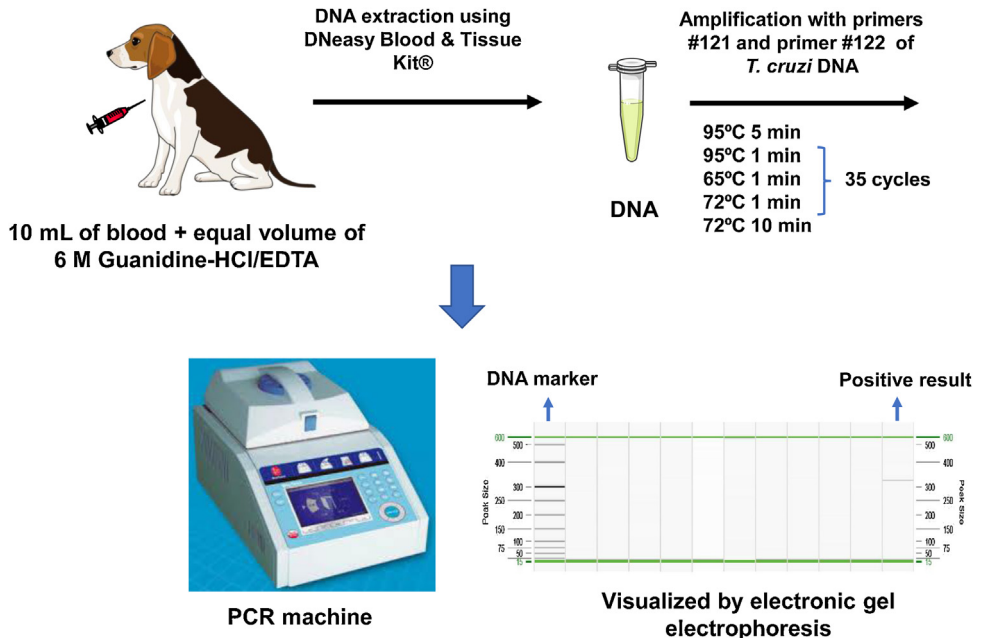
Fig. 2. Hemoculture (HC).

Fig. 3. PCR in blood eluate.

culture medium [5] through centrifugation under the same conditions and the supernatant was discarded. The leukocyte layer was transferred to a conical cell culture tube and LIT medium was added (5 mL). The red cell layer was divided equally into two tubes with equal volumes of LIT. All tubes were incubated in a BOD chamber at 28 °C and homogenized every 48 h. One drop of the pellet from each tube was analysed under the optical microscope at 30, 60, 90 after culture for the detection of parasites. Negative tube were examined again at 120 days, followed by centrifugation at 1500 xg for 10 min at 4 °C, the culture discarded and the sediment examined under the microscope. This method, although of lower sensibility than PCR or qPCR is very common after treatment because it clearly demonstrates treatment failure via detection of the parasite.

PCR in blood eluate

For this test illustrated at Fig. 3, 10 mL of venous blood from each dog collected from the cephalic vein were immediately transferred to conical plastic tubes with 10 mL of 6 M Guanidine-HCl/0.2 M EDTA (Sigma® Chemical Company, USA) at pH 8.0 [6]. This mixture was maintained at room temperature for seven days, boiled in a water bath at 100 °C for 10 min [7] and the lysate was stored at room temperature. DNA was extracted from a 2-mL aliquot of the sample blood lysate using the "DNeasy Blood & Tissue Kit" kit (Qiagen Inc., USA) and the extraction protocol according to the manufacturer's instructions. For DNA amplification, the primers # 121 (AAATAATGTACGGGTGA-GATGCATGA) and # 122 (GGTTCGATTGGGGTTGGTGTAATATA) (Invitrogen, São Paulo, Brazil) were used for amplification of the variable region of the *T. cruzi* kDNA 330 bps minicycles. Amplification of the dog constitutive gene was carried out as reaction control using the primers for amplification of the GAPDH gene: Fw (5 'CTA CCC ACG GCA AAT TCC 3') and Rw (5 'ACT CAG CAC CAG CAT CAC 3'), which amplify a 129 bp product (GENBANK: AB038240.1). The reaction mixture for PCR consisted of: 6.5 pmol of each primer (# 121 and # 122) or 2.5 pmol (GAPDH), 6.25 μl GoTaq® Green Master Mix 2X (Promega, Madison, USA) and 5.0 μL DNA and MilliQ Water in a total volume of 15.0 μL per well on a 96-well plate (MicroAmp® Fast Optical 96-Well, Applied Biosystems). The conditions used in the PCR reaction were adapted from Gomes et al. [8]: initial denaturation at 95 °C for 5 min, followed by 35
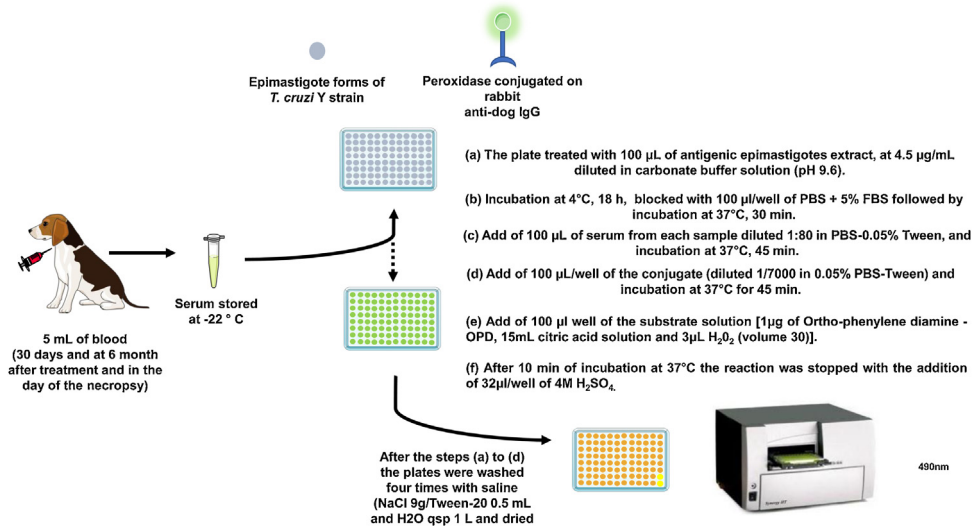
Fig. 4. Conventional Serological Test - Enzyme-linked-immunosorbetn-assay (CS-ELISA).

cycles at 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. The equipment used was a thermal cycler Verit Termal Cycler 96-well (Applied Biosystems®, California, USA). Visualization of the amplified products were performed using the QIAxcel Advanced System® capillary electrophoresis apparatus (Qiagen), AM320 analysis method, 15 bp / 600 bp alignment marker (QX Alignment Marker®, Qiagen) and 25 bp molecular weight marker / 500 bp (QX Size Marker® Qiagen), according to the manufacturer's recommendations for the sizes of the fragments analyzed. After electrophoresis, the presence/absence of bands in the regions of interest of the electropherogram were obtained through QIAxcel ScreenGel 1.2.0® software (Qiagen). The contamination hypothesis was eliminated by using a negative control of the reaction and of the extraction, as well as a control of extraction reagents and PCR. PCR in blood eluate as here employed offers evidence of the presence of kDNA of *T. cruzi* blood trypomastigotes, alive or dead, in treated animals. However, its negativation may not be interpreted as absence of infection, as infection may be restricted to the host tissues. All samples were processed in duplicate in parallel to positive and negative controls.

Conventional serological test - Enzyme-linked-immunosorbent-assay (CS-ELISA)

Blood samples (5 mL) were collected from the cephalic vein of dogs 30 days and twice a year after treatment and on the day of the necropsy. The serum was stored at -22 °C until further examination. The different steps of the CS-ELISA are shown in Fig. 4. Alkaline antigen of epimastigote forms of *T. cruzi* Y strain obtained from acellular culture in LIT medium [5] at exponential phase of growth [9] and peroxidase-labeled anti-dog IgG immunoglobulins conjugate (Bethyl Laboratories, Montgomery, USA) were used. The ELISA assay was carried out according to the methodology described by Voller et al. [10], modified according to Santos et al. (2012) [11]. Each well of the plate was treated with 100 μL of total antigenic epimastigotes extract followed by protein dosage [12], The concentration previously defined by block titration was 4.5 μg/mL, diluted in carbonate buffer solution (pH 9.6). The plates were incubated at 4 °C for 18 h and blocked with PBS + 5% FBS (100 μL / well), followed by incubation at 37 °C for 30 min. The next step consisted of addition 100 μL of serum from each sample diluted 1:80 in PBS with Tween 0.05% (PBS-Tween), and incubated at 37 °C for 45 min. Subsequently, the conjugate (100 μL/well, diluted to 1/7000 in PBS-Tween as per the previous titration), was added and the plates were incubated at 37 °C for 45 min. Following, the substrate solution (1 μg of ortho-phenylene diamine-OPD, 15 mL citric acid solution and 3 μL H_2O_2 (30%), 100 μL/well) were added, incubated for 10 min at
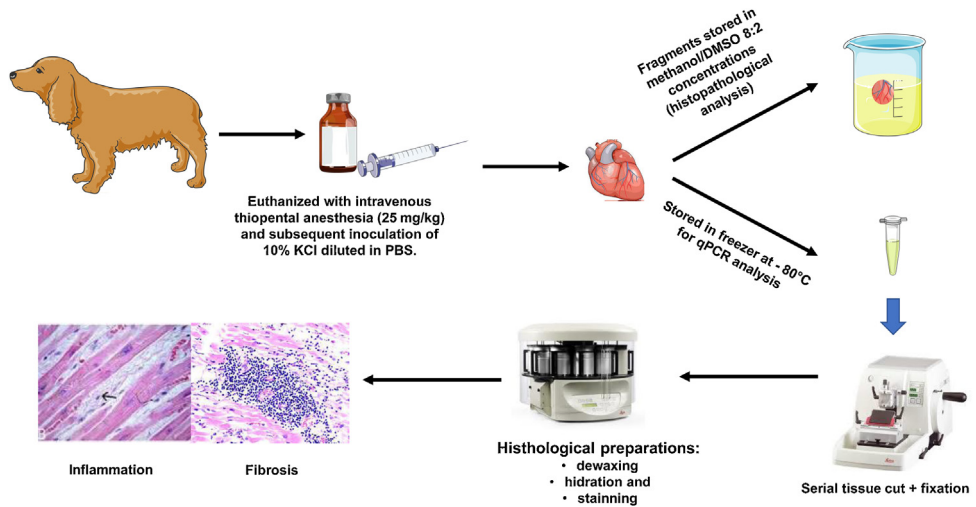
Fig. 5. Necropsy and histopathology.

37 °C and the reaction was stopped with the addition of 4 M H_2SO_4 (32 µL / well). After each step the plates were washed four times with saline (NaCl 9 g/Tween-20 0.5 mL, and H_2O qsp 1 L). The reaction reading was performed on a microplate reader (BIO RAD, Model 680) with a 490 nm filter. Ten negative control sera and four positive controls were added to each plate. The discriminant absorbance in each plate was calculated by taking the mean absorbance of the ten negative sera plus two standard deviations. Sera with OD (optical density) values below the cut-off were considered negative and samples above the cut-off were considered positive. All sera samples were examined in duplicate.

In treated hosts negativation of the serological test together with consistently negative parasitological examinations (HC, PCR, qPCR) is considered evidence of cure (classical cure criteria according to the Second Brazilian Consensus on Chagas Disease [3]). This interpretation is practical for applications in public health but has been under discussion by parasitologists and immunologists. This is because, in spite of repeatedly negative parasitological tests, the residual positivity of serological tests (ELISA or others) may be due to the interplay of several other factors and may not necessarily be a sign of active *T. cruzi* infection [13]. Therefore, treated hosts with a decrease in serological tests reactivity (decay of anti-*T. cruzi* IgG) together with consistently negative parasitological tests are interpreted as "in the process of cure" and need further evaluation with serological and parasitological tests.

Necropsy and histopathological analysis

Necropsy and histopathology are represented in Fig. 5. Necrospy was performed in dogs that died naturally throughout the infection and in the end of the study at 18 months. The animals were euthanized with thiopental anesthesia (at least 25 mg/kg, intravenous, until obtaining an adequate anesthetic plane III to IV) and subsequent application of 10% KCl diluted in PBS. During the necropsy, fragments of the heart (right atrium and left ventricle) were sectioned and collected. Part of these fragments were stored in cassettes using methanol: DMSO (80:20 v/v) for further histological evaluations. The rest of the fragments were wrapped in aluminum foil and stored at −80 °C for further molecular parasitological evaluation by qPCR.

Histopathological analyses of heart fragments (right atrium and left ventricle), 4 µm tissue sections from the same area, examined by qPCR). The cardiac tissue was chosen because the VL-10 *T. cruzi* strain has special tropism for the heart in mice model and because the regions selected show the presence of severe parasitism according to previous reports [14]. Serial cuts were dewaxed in xylene immersions, hydrated in alcoholic solutions of decreasing concentrations, and washed in running water and PBS. The cardiac inflammatory infiltrate was evaluated in Hematoxylin & Eosin
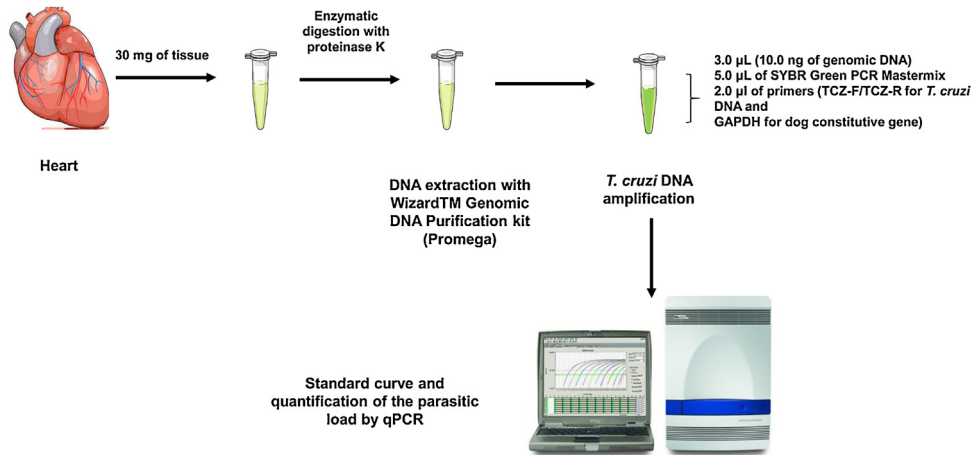
Fig. 6. Molecular parasitological evaluation (qPCR) in cardiac tissue.

(H & E) staining. The slides were analyzed under an optical microscope (Leica DM5000B). Inflammation analysis was performed using the Leica QWin software (Leica Microsystems, Wetzlar, Germany). Morphometric studies involved the analysis of 20 randomly selected fields (total area $1.5 \times 10^6\ \mu m^2$) of tissue sections on a single slide per animal. Inflammatory cells were quantified in the cardiac fragments by count of the cell nuclei present. The difference between the number of cell nuclei present in the infected animals and that observed in uninfected animals determined the normal pattern of inflammatory cells. Data were expressed as mean number ± standard deviation of nucleated cells, as described by Maltos [15]. Collagen deposition was carried out by qualitative comparative analysis in tissues stained with Masson trichrome stain. The examination was performed under optical microscopy (40×). Myocardial fibrosis was evaluated using an optical microscope (Olympus CH30, Japan). Semi quantitative histological analysis was performed by means of scores on a scale of 0, 1, 2 and 3 corresponding to absence, discreet, moderate and intense presence of fibrosis, respectively, according to Iordanou et al. [16], modified. The histopathological evaluation shows the presence of the two important pathological processes present in Chagas disease (inflammation and fibrosis) and responsible for the morphophysiological changes and their clinical consequences.

Molecular parasitological evaluation (qPCR) of cardiac tissue

The quantitative polymerase chain reaction (qPCR) technique was performed according to the protocol standardized by Caldas et al. (2012) [17] and is represented in Fig. 6. This technique aims to quantify the tissue parasitism in the animals' hearts 18 months after treatment. The heart was chosen because it was previously demonstrated that the VL-10 *T. cruzi* strain has special tropism for this organ [14].

The fragments were cut with a knife and scalpel, washed with PBS, and weighed (30 mg each). The samples were transferred to 1.5 mL tubes and the total DNA extraction procedures were performed using the WizardTM Genomic DNA Purification kit (Promega) following the manufacturer's recommendations with some modifications. Initially, nuclear lysis solution (500 u L) was added and the tubes were kept on ice for 2 min. After homogenization by inversion 20.0 μL of Proteinase K (Sigma-Aldrich®, USA) at a concentration of 20.0 mg/mL were added in the tubes, then 3 μL of RNAse were added and the samples were incubated for 30 min in a 37 °C dry bath. After incubation the tubes were kept at room temperature for 5 min and 200 μL of the protein precipitation solution was added. The samples were vortex-mixed (Certomat MV, B. Braun Biotech International, USA) homogenized for 20 s and centrifuged for 5 min at 16,000 × g (Eppendorf Microcentrifuge Model 5418, NY, USA). Thereafter, the supernatant was transferred to another tube containing 600 μL of isopropanol (Merck®, Darmstadt, Germany). This tube was then homogenized and centrifuged for 1.5 min at

16,000 × g. The supernatant was discarded and 70% ethanol (Merck®, Darmstadt, Germany, 200 µL) was added to the pellet. Subsequently a new centrifugation was performed at 16,000× g, and the supernatant discarded again. The tubes were kept open for evaporation of the remaining ethanol and then 100 µL of the hydration solution were added and the DNA left to rehydrate for 24 h at room temperature. Next, the concentration and quality of the DNA obtained were determined by spectrophotometry (Nanodrop, GE Healthcare Products).

To quantify the tissue parasitism in the fragments by qPCR, a standard curve was built to determine the number of copies of the parasite DNA in the sample. For this, epimastigote forms of the *T. cruzi* Y strain grown in LIT medium were used until reaching the total number of 1.0×10^8 parasites counted in a Neubauer chamber, which were submitted to the DNA extraction protocol described above. After dilution of the extracted DNA pellet in autoclaved ultrapure water (100 mL), the concentration obtained was 10^6 parasites/mL, assuming that the extraction was 100% efficient. Serial dilutions of the DNA were performed in autoclaved ultrapure water (1:10) to obtain seven spots on the curve ranging from 10^6 to 1.0 parasite. Each sample was analyzed in duplicate for amplification of *T. cruzi* DNA and in each qPCR reaction 3.0 µL of the extracted and diluted sample were used (containing 10.0 ng of genomic DNA), 5.0 µL of SYBR Green PCR Mastermix "(Applied Biosystems) and 2.0 µL of primers. The primers used for the amplification of the *T. cruzi* DNA were: TCZ-F 5′-GCTCTTGCCCACAMGGGTGC-3′, wherein M = A or C, and TCZ-R 5′ - CCAAGCAGCGGATAGTTCAGG-3′; to amplify a product of 182 bp [18]. For each sample, a reaction was also performed to measure the dog constitutive gene (GAPDH), used as endogenous control, using 3.0 µL of the extracted sample (10.0 ng of genomic DNA), 5.0 µL of SYBR Green PCR Mastermix "(Applied Biosystems) and 2.0 µL of primers. The primers for the GAPDH were: Fw (5 'CTA CCC ACG GCA AAT TCC 3′ and Rw (5 'ACT CAG CAC CAG CAT CAC 3′), and a 90 bp product was amplified (GENBANK: AB038240.1). The reaction was always carried out in parallel to negative controls, which were also submitted to the reaction with the specific primers of *T. cruzi* and the dog GAPDH constitutive gene, including wells with specific *T. cruzi* and water (duplicate for *T. cruzi* and in unicata for GAPDH). The reactions were distributed in 96-well plates (Fast 96-Well Reaction Plate, 0.1 mL, MicroAmp ™). The samples were then subjected to initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 63 °C for 1 min. qPCR, applied to host tissues is a high sensitivity method and is very important in post-treatment evaluation. qPCR offers complementary information to PCR in blood eluate because it can demonstrate the presence of kDNA of *T. cruzi* (alive or dead) in the host tissues, referent to the amastigote stage of the parasite.

Resource availability

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