Molecular and biological characterization of a highly pathogenic *Trypanosoma cruzi* strain isolated from a patient with congenital infection

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PII: S0014-4894(17)30495-2
DOI: 10.1016/j.exppara.2018.02.002
Reference: YEXPR 7521

To appear in: *Experimental Parasitology*

Received Date: 5 September 2017
Revised Date: 23 November 2017
Accepted Date: 11 February 2018


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Congenital case of Chagas disease

T. cruzi isolated from newborn

Genetic characterization

In vitro drug sensibility

In vivo characterization and treatment response

T. cruzi VD strain Characterized and suitable for experimental studies
Running title: *T. cruzi* VD strain characterization

Molecular and biological characterization of a highly pathogenic *Trypanosoma cruzi* strain isolated from a patient with congenital infection

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Abstract

Although many Trypanosoma cruzi (T. cruzi) strains isolated from a wide range of hosts have been characterized, there is a lack of information about biological features from vertically transmitted strains. We describe the molecular and biological characteristics of the T. cruzi VD strain isolated from a congenital Chagas disease patient. The VD strain was typified as DTU TcVI; in vitro sensitivity to nifurtimox (NFX) and beznidazole (BZ) were 2.88 µM and 6.19 µM respectively, while inhibitory concentrations for intracellular amastigotes were 0.24 µM for BZ, and 0.66 µM for NFX. Biological behavior of VD strain was studied in a mouse model of acute infection, resulting in high levels of parasitemia and mortality with a rapid clearance of bloodstream trypomastigotes when treated with BZ or NFX, preventing mortality and reducing parasitic load and intensity of inflammatory infiltrate in skeletal and cardiac muscle. Treatment-induced parasitological cure, evaluated after immunosuppression were 41% and 35% for BZ and NFX treatment respectively, suggesting a partial response to these drugs in elimination of parasite burden. This exhaustive characterization of this T. cruzi strain provides the basis for inclusion of this strain in a panel of reference strains for drug screening and adds a new valuable tool for the study of experimental T. cruzi infection.

Keywords
Trypanosoma cruzi; Chagas’ disease; Congenital transmission; Discrete Typing Unit
Benznidazole; Nifurtimox.

Funding:
Agencia PICTO-GLAXO 2012-035, UBACyT 2013-065BA and 2016-240BA.
1. Introduction

American trypanosomiasis is an anthropozoonosis caused by the hemoprotozoa parasite *T. cruzi*. Also known as Chagas disease, it is endemic in Latin America, with at least 5-6 million people infected, 70 million people at risk of infection and 100,000 deaths per year [1].

In endemic areas, the highest incidence of *T. cruzi* infection occurs in children under 10 years old [2]. Based on seroprevalence studies in pregnant women, it is estimated that nearly 1,400 children are born congenitally infected each year in Argentina, where 1.6 million infected people live [1]. Remarkably, vertical transmission is currently responsible for most new cases in urban areas [3].

The life cycle of *T. cruzi* is complex and involves over 150 mammalian hosts and triatomine bloodsucking bugs. Transmission occurs when infected bug feces contaminate the bite site or intact mucous membranes, through blood transfusion, organ transplants, or transplacental passage. Oral infection linked to ingestion of food and/or drinks contaminated with infected triatomine feces has been also recognized as a source of outbreaks in the past few years [4].

Treatment of Chagas disease currently relies on two antiparasitic drugs: benznidazole (BZ) and nifurtimox (NFX). Both compounds have an estimated efficacy of 70 and 98% in acute and congenital cases, respectively, but effectiveness decreases for patients in the chronic phase [3].

Over the past few years, a consensus was reached towards the classification of *T. cruzi* strains into six discrete typing units (DTUs), defined as “set of stocks that are genetically more related to each other than to any other stock and that are identifiable by common genetic, molecular, or immunological markers”, designated as Tc I to TcVI [5]. *T. cruzi* intraspecific genetic and phenotypic diversity was widely characterized by biological behavior, biochemical proprieties and pharmacological response [6].

*T. cruzi* diversity can also be observed in experimental infections and treatment response. Several isolates from differente sources (humans, domestic and sylvatic animals and bugs) have been characterized *in vitro* and in experimental animal models, including response to available drugs [6–9]. Although there are some reference strains employed for experimental
studies, there is a lack of information on biological behaviour, drug sensitivity and treatment response for *T. cruzi* strains isolated from transplacental infection.

The aim of this work was to characterize the biological and molecular properties of a *T. cruzi* isolate, the VD strain, obtained from a pediatric congenital case.

2. Materials and Methods

2.1 Clinical case

A three months old baby born in a non-endemic area to a previously non treated mother with chronic indeterminate phase of Chagas disease, was referred to the Service of Parasitology and Chagas Disease, "Dr. Ricardo Gutiérrez" Children’s Hospital (Buenos Aires, Argentina) with low weight, jaundice, hepatosplenomegaly and positive *T. cruzi* parasitemia. The child was treated initially with BZ but interrupted due to gastric intolerance; treatment was changed to NFX with good tolerance and clinical response. After treatment completion, the patient returned to his hometown and was lost to follow up.

2.2 Strain isolation

A blood sample from the pediatric patient was inoculated subcutaneously in 14 days old Rockland mice (n=6) (González-Cappa, SM, laboratory records). Parasites obtained from this initial isolate have been maintained by weekly passages in 21 days old CF-1 male mice, infected with $1 \times 10^5$ bloodstream trypomastigotes by intraperitoneal (ip) route. Bloodstream trypomastigotes are also routinely stored in liquid nitrogen. This *T. cruzi* isolate will be referred to as VD strain. For experimental purposes, VD strain bloodstream trypomastigotes were purified from heparinized mice blood as previously described [10].
2.3 Compounds and reagents

For *in vitro* assays, pure powder of BZ (N-Benzyl-2-nitroimidazole acetamide) (provided by Elea®, Buenos Aires, Argentina) and NFX (3-methyl-4- (5′-nitrofurfurylideneamino) tetrahydro-4H-thiazine -1,1-dioxide) (provided by Bayer®, Germany) were suspended in dimethylsulfoxide (DMSO). For *in vivo* assays, BZ (Abarax®, Elea) and NFX (Lampit®, Bayer) tablets were pulverized and resuspended in a 0.25% carboxymethylcellulose (CMC) solution (Sigma-Aldrich, USA).

2.4 *In vitro* assays

2.4.1 Cell culture

Vero cells were grown in 25 cm² tissue-culture flasks, using L-glutamine RPMI-1640 medium supplemented with 5% of FCS, 10 mM HEPES, 100 UI/mL penicillin and 50 µg/mL streptomycin, and incubated in 5% CO₂ atmosphere at 37°C.

2.4.2 Trypomastigotes drug susceptibility

VD strain trypomastigotes purified from mice blood were seeded in 96-wells polystyrene flat bottom plates in 100 µL of RPMI-5% FCS (1x10⁸ parasites/mL), exposed to five serial dilutions of NFX (100-0.3 µM) or BZ (230-0.1 µM) and incubated at 37°C in a 5% CO₂–95% air mixture for 24 hours. Motile parasites were counted in a Neubauer chamber. Each drug concentration was evaluated by triplicate and compared to untreated control cultures. The 50% lytic concentrations (LC₅₀), defined as the drug concentration that resulted in a 50% reduction of trypomastigotes viability compared to the non-treated controls, was estimated by non-linear regression analysis [11].
2.4.3 Amastigote growth inhibition assay

Cesium 137 irradiated Vero cells (2000 rad) were plated overnight at 37°C-5% CO₂ to a density of 5x10⁴ of cells/well, in 24-well plates with rounded coverslips at the bottom. Infection conditions were previously optimized with 2.5x10⁵ parasites/well and incubated for 3 hours at 37°C. Then, wells were washed with PBS to remove non-attached parasites and fresh RPMI media containing NFX (20 to 0.2 µM), BZ (10 to 0.16 µM) or medium was added. Drug-containing media was renewed every 24 hours for 3 consecutive days; Then, cells were washed with PBS, fixed with methanol and stained with Giemsa. Each drug concentration was evaluated by triplicate. The 50% inhibitory concentration (IC₅₀), defined as the drug concentration required to achieve 50% inhibition of intracellular amastigote growth by counting number of amastigotes per cell, was determined by non-linear regression analysis [11].

2.5 In vivo assays

2.5.1 Animal breeding conditions

Twenty days old female BALB/c mice (15 ± 2 grams) were obtained from the Animal Facilities at the Faculty of Veterinary Sciences, University of Buenos Aires (Argentina) and maintained under conventional closed barriers at the "Dr. Ricardo Gutiérrez" Children’s Hospital Animal Facilities. Animals were housed in 600 cm² polycarbonate at 4-5 animals per cage. Mice were individually identified and cages were properly labelled. Cages were filled with irradiated chip-bedding and changed once a week. Mice had access to food and water ad libitum. Macroenvironmental conditions included a 12:12 hs light:dark cycle (starting at 6 a.m.), controlled temperature of 20 to 22 °C, and 45% to 55% humidity.
2.5.2 Infection and treatment

Mice were infected at 5 to 7 weeks of age (16.25 ± 2.09 grams) by the intraperitoneal route (ip) with 500 bloodstream VD strain trypomastigotes. Blood direct microscopic observation was performed starting at the 5\textsuperscript{th} day post infection (dpi). Treatment was begun when parasites were detected in circulation; animals were then randomly assigned to the following groups: BZ (n = 17), NFX (n = 17) or infected non-treated (NT, n = 17).

Treatment was administered orally for 20 consecutive days at a dose of 100 mg/kg/day for NFX or BZ, using disposable tips and an automatic pipette, in a final volume of 50 µL per animal per dose. The doses, length and route of administration were chosen based on previous published literature [12,13].

Parasitemia was evaluated three times a week during therapy by pricking the tail and collecting 5 µl of fresh blood. Blood was incubated with red blood cell lysis solution (20 µl of Tris-buffered 0.83% ammonium chloride pH 7.2), and motile parasites were counted in a Neubauer chamber. In addition, the following parasitemia parameters were recorded: prepatent period (defined as time between infection and detection of circulating parasites), patent period (time elapsed between parasitemia onset and negativization), parasitemia peak (highest parasitemia level), and number of drug doses needed to turn parasitemia undetectable. Animals were periodically weighed to adjust doses and to assess changes in body weight. Body temperature was determined rectally with a pediatric digital thermometer. Mortality was recorded daily. To avoid unnecessary pain and stress, pre-established anticipated endpoints were used, and animals were euthanized if they fulfilled any of these criteria (i.e. 20\% weight loss from initial body weight, body temperature lower than 33.5°C, or parasitemia higher than 2x10\textsuperscript{6} trypomastigotes/mL). Euthanasia was performed with CO\textsubscript{2} inhalation in a saturated chamber or sodium pentothal overdose (300 mg/kg, ip).
2.5.3 Determination of parasitological cure

At the end of the treatment, 5 surviving animals from each group were euthanized, and blood and tissue samples were obtained for *T. cruzi* DNA detection by real-time PCR (qPCR) and histopathology. Remaining animals with undetectable parasitemia were left without treatment for 10 days, with periodic checks for re-emergence of bloodstream parasites, and then subjected to a cyclophosphamide (CYP)-based immunosuppression protocol to rule out parasite reemergence from sanctuary sites in tissues [14]. Briefly, CYP was given once a week by ip injection at 200 mg/kg for a total of 4 administrations. Animals were euthanized if parasite ressurgence was observed or, if not, after four cycles of immunosuppression, tissues (heart, skeletal muscle and brain) and blood were collected for confirmation of cure by histopathology and qPCR.

2.6 Tissue sample preparation and histological evaluation

Samples from heart, skeletal muscle, brain, liver, kidney, colon and uterus were collected from all animals to evaluate inflammation and presence of amastigote nests. Samples were fixed in buffered 10% formaldehyde, dehydrated, paraffin-embedded, and 5 µm thick sections were stained with haematoxylin and eosin (H&E).

Evaluation of the tissue specimens was performed by light microscopy by a researcher blinded to the treatment assignment. Presence of amastigote nests was recorded and the degree of myocardial and skeletal inflammation was scored as described previously [15,16]. General histoarchitecture, inflammatory infiltrates, degree of vascular congestion and presence of amastigote nests were evaluated in liver, kidney, colon, uterus and brain. Number of inflammatory foci was determined in 100 fields at 400X magnification and extent of infiltration (expressed in µm²) was measured using Infinity Analyse program 6.5.0 (Lumenera Corp®).
2.7 Quantitative PCR (qPCR)

Blood samples were collected and diluted 1:3 in guanidine-EDTA buffer (GEB) (6M guanidine-HCl, 0.2M EDTA), and tissue (heart, skeletal muscle and brain) samples were rinsed with sterile distilled water and stored at -70°C until processing. DNA was extracted with High Pure PCR Template Preparation Kit (Roche®) according to the manufacturer and stored at -70°C until use. The extracted DNA was quantified by spectrophotometry at 260 nm wavelength with Nanodrop 1000 (ThermoScientific®). 

*T. cruzi* DNA amplification was performed using *cruzi* 1 [5'-3'-ASTCGGCTGATCGTTTTCGA] and *cruzi* 2 [5'-3'-AATTCCTCCAAGCAGCGGATA] primers, which amplify a 166-bp specific fragment, corresponding to satellite DNA. The detection was performed using TaqMan® probe *cruzi* 3 [5'-3'CACACACTGGACACCAA] as previously described [17]. The standard curve allowed quantification of DNA between a range of 1.6x $10^0$ to 8x$10^5$ parasite equivalents/mL. An internal amplification standard (IAC) of DNA extraction (2 ng) was included in each GEB sample.

2.8 Discrete Typing Unit (DTU) determination

Using purified DNA from cell-culture derived trypomastigotes, the DTU of the VD strain was identified based on the molecular weight of the bands amplified by PCR targeting specific nuclear gene sequences: the intergenic region of spliced leader genes (SL-IR), the 24Sα subunit ribosomal DNA (rDNA 24Sα) and the A10 fragment as previously described [18]. Amplification products were stained with Gelred® (Genbiotech SRL) and visualized in a 3% agarose gel.
2.9 Statistical Analysis

For *in vivo* studies, data was subjected to analysis of variance test (ANOVA) with post-hoc correction for multiple comparisons with Bonferroni test or non-parametric Kruskal-Wallis test, and compared in pairs depending on data distribution. Survival analysis was performed using Kaplan-Meier test. In all cases, p-values<0.05 were considered statistically significant. Statistical analyses for *in vivo* assays were performed with InfoStat/P 2014 (Universidad Nacional de Córdoba, Argentina). BZ and NFX LC\(_{50}\) and IC\(_{50}\) were determined by non-linear regression using GraphPad Prism 5.03 software (GraphPad Software, Inc. USA). Graphics were prepared with GraphPad Prism 5.03. Values in tables and graphs are expressed in mean values with standard deviation unless otherwise indicated.

2.10 Ethical statement

Animals were acclimatized to new housing conditions and habituated to routine handling by trained personnel for two weeks prior to the experiment and procedures were performed according to local guidelines [19]. Protocol was approved by the Institutional of Animal Care and Use Committee from Faculty of Veterinary – University of Buenos Aires (# Protocol: 2014/4). Anticipated endpoints (see above) were implemented to avoid unnecessary suffering.

3. Results

3.1 Discrete Typing Unit (DTU) determination

DNA analysis from cell-culture derived VD strain trypomastigotes revealed that it belongs to the DTU TcVI according to SL-PCR II 425 bp, heminessed 24αS ribosomal PCR 140 bp and A10 fragment 630 bp (Supplementary Material, SM 1).
3.2 In vitro drug sensitivity

VD strain susceptibility to reference drugs (i.e. BZ and NFX) both in trypomastigotes (LC₅₀) and intracellular amastigotes (IC₅₀) is shown in Table 1 and dose-response curves are displayed in Supplementary Material 2 (SM2).

3.3 Course of acute infection with T. cruzi VD strain in a murine model

Parasites were detected in mice blood at 8 (range 5 to 9) days post infection (dpi) (i.e. afterip inoculation), at which point they were divided into treatment (NFX or BZ) or NT groups. Mice from the NT group developed high levels of bloodstream VD trypomastigotes (Figure 1A), unlike BZ or NFX groups which showed treatment response with a rapid decrease in bloodstream parasites. Parasitemia parameters are shown in Table 2.

BZ and NFX were well tolerated throughout the study by all treated mice. Animals treated with BZ or NFX were clinically healthy, with shiny and well groomed fur, showing alert behavior, connected with environment. In contrast, animals in the NT group failed to recover clinically, showing a soiled and bristly coat, with hunched guarding posture and partially closed eyelids accompanied by tearing. Reduced mobility with lethargic reflexes and decreased awareness were also observed. These clinical signs were accompanied by weight loss and decreased body temperature, reaching the maximum pre-established tolerable endpoint near peak parasitemia, at approximately 21 dpi (Figure 1B).

None of the animals in the NT group gained body weight during follow up, reaching weight nadir at 21 dpi. On the other hand, animals treated with BZ or NFX exhibited a less pronounced weight loss, especially in the NFX group, and a rapid weight recovery during the treatment period. Moreover, animals from the NT group exhibited lower average body temperature compared to NFX or BZ treated animals, starting from 12 dpi and reaching values closely to hypothermia at approximately 21 dpi, coinciding with peak parasitemia levels (Supplementary Material SM3).
3.4 Histopathological features

The skeletal muscle in all studied animals in the NT group showed a high degree of rhabdomyolysis with mononuclear inflammatory infiltrates giving a mean inflammatory score of 3.5 over 5 and muscle fiber calcifications were observed in 4 samples. Amastigote nests could be observed in 82.35% of animals (14 / 17). By contrast, animals treated with BZ or NFX exhibited comparatively lower degrees of inflammation, with scores ranging from 0.5 to 3 (over 5), and mild to moderate interfibrillar mononuclear inflammatory infiltrates without rhabdomyolysis (Figure 2A). Amastigote nests were observed in 4 of 17 samples from BZ group, whilst specimens from NFX-treated animals had no detectable amastigote nests.

Animals from the NT group also showed myocardial inflammatory foci, in some cases with local extension without involving complete heart wall (inflammation score=3) with predominantly mononuclear cells infiltrates. Inflammation scores ranged from 1 to 3 (over 5). Also, 65% (11 / 17) of NT mice showed amastigote nests within cardiac fibers. BZ-treated animals showed greater variability in degree of inflammation, with scores ranging from 0 to 4, with focal, but not confluent, infiltrates in the peripheral heart wall being the predominant finding. Only 29.5% (5/17) BZ-treated animals exhibited amastigote nests in myocardial samples. Mice from the NFX group showed myocardial inflammation ranging from 0 to 3 points (over 5). Similarly to BZ-treated animals, focal lesions with mononuclear infiltrates were predominant. However, amastigote nests were observed only in one animal (6%; 1 / 17). (Figure 2B)

Focal mononuclear infiltrates were also the main feature in liver samples from all experimental groups. No differences were observed among groups in the number of inflammatory foci, but their sizes were larger in NT animals, followed by those treated with BZ (Figures 2C-2D). Amastigote nests were not identified in liver histological sections from any experimental group.

No significant morphological and/or histological alterations, nor amastigote nests were observed in kidney, colon, uterus and brain samples of any experimental group.
3.5 Assessment of treatment-induced parasitological cure

After completion of 20 consecutive days of therapy with either BZ or NFX, 5 animals from each group were euthanized to obtain blood samples and target organs (skeletal muscle, heart and brain) in order to analyze parasite burden by qPCR. Table 3 shows percentage of animals with parasitic sterilization (without immunosuppression) in each treatment group (i.e. BZ or NFX). Estimated parasitic loads are illustrated in Figure 3. Parasitological cure rates in BZ or NFX treated animals after immunosuppression with CYP are shown in Table 4.

In summary, adding all negative animals irrespective of whether they received immunosuppression or not, the overall parasitological cure was observed in 41% (7/17) of the BZ-treated animals, and in 35% (6/17) of the NFX-treated animals.

4. Discussion

We have conducted the first exhaustive description of the main biological and molecular characteristics of the *T. cruzi* VD strain, originally isolated by our group from a congenital Chagas disease case. Based on well-established DTU methods, the VD strain belongs to DTU TcVI, supporting previous findings [20] and suggesting genetic stability through mice passages. DTU TcVI is one of the most prevalent *T. cruzi* genotype in domestic transmission cycle and it have been linked to congenital cases [18]. *In vitro* susceptibility to BZ and NFX was assessed both in bloodstream trypomastigotes and amastigotes. Inhibitory concentration values for BZ and NFZ were similar to those reported from *T. cruzi* reference strains within DTU Tc VI (Tulahen and CL-Brener), but considerably below the lytic concentration from Tulahuen trypomastigotes (Table 1). Unfortunately, comparison with NFX *in vitro* sensitivity on *T. cruzi* trypomastigotes was hampered because most LC$_{50}$ were established on epimastigote stage, which does not represent the same biological and clinical characteristics of the trypomastigote and amastigote stages, including drug sensitivity to reference drugs.
Given the extreme variability in drug sensitivity even within the same DTU, there is no convincing evidence yet to support the association between *in vitro* sensitivity to conventional trypanocidal drugs and phylogenetic features. There are also no consistent data to support any correlation between DTU and morbidity, risk of reactivation or congenital transmission in humans [3].

While many animal models have been developed for studying Chagas disease [21], a murine model was chosen to characterize the VD strain due to the common use of this animal species for isolates maintenance, biological characterization and as suggested model for initial evaluation of tripanocidal compounds [12]. BALB/c mice strain were chosen due to their previously recognized high susceptibility to different *T. cruzi* isolates. Although host variables such as age and sex may influence the course of the experimental infection [21], female mice were preferred due to easier handling and the selected age allowed induction of patent parasitemia in all animals, at a similar time (8 days post-infection) with circulating parasites counts in the same order of magnitude. However, trials with new drugs may consider evaluating efficacy in both sexes in order to improve predictability.

Experimental mice infection with highly virulent *T. cruzi* strains (such as VD strain) produce elevated parasitemia and high mortality rates. These two effects can allow quick and easy determination of parasiticidal activity of new compounds, and make VD strain very suitable for acute infection models for drug screening [12]. The chosen inoculum was comparatively low compared to previous works [22,23], but it was based on the high virulence that VD strain exhibited. At the end of the acute phase of the infection, untreated mice mortality was near 95% in our model. These values are consistent with previous characterization of *T. cruzi* virulent strains [9,24].

Likewise, oral administration of BZ and NFX produced a rapid decline in parasitemia. These results illustrate an effective treatment response with conventional drugs consistent with previous descriptions [23].

In our model, parasitemia reached maximum values by 18 dpi in NT animals, accompanied by symptoms compatible with systemic infection and reduced weight and body temperature. Weight loss is typical of the experimental acute phase and could be related to the lower average water and food consumption.
Histopathological findings during the acute phase of infection showed a marked tropism for skeletal and cardiac muscle. NT animals were significantly more likely to have amastigote nests observed in these tissues than those treated with BZ and NFX, even in those who underwent immunosuppression. These results contrast with observations from models with CL-Brener or RA strains (both DTU TcVI) with a marked tissue pantropism [25,26].

Morphometric analysis of liver samples showed larger inflammatory areas in NT animals compared to those treated with conventional drugs. However, treatment with BZ and NFX was unable to decrease concomitant liver inflammation. Future studies should focus on defining whether this infiltrate is a consequence of parasite infiltration, the toxic action of nitroheterocyclic drugs or due a combined effect between *T. cruzi* infection and treatment, as suggested previously [27].

Absence of amastigote nests in liver agrees with previous data from isolates from *T. rubrovaria* (DTU TcIII) in female Swiss mice [28] and might be related to the involvement of this organ as a major site of immunological elimination of parasites [29].

An immunosuppression protocol with CYP and and qPCR quantification of parasite load in blood and target tissues were chosen to determine parasitological cure. The immunosuppression protocol proved to be a good marker for treatment failure, as all animals showing parasite DNA in at least one of the analyzed tissues subsequently reactivated after administration of CYP.

Importantly, none of the BZ treated animals showed persistent parasitemia by fresh blood examination after end of therapy, while only one mouse treated with NFX showed blood trypomastigotes after 20 consecutive days of treatment. Two heart and skeletal muscle samples from NFX-treated animal which did not reactivate after immunosuppression were positive by qPCR, but due to their low parasitic load could not be quantified. Interestingly, DNA was not detectable by qPCR in blood of these mice. The presence of residual DNA in tissues or possible PCR cross contamination could explain these non quantifiable positive levels of *T. cruzi* DNA in tissues. Guarner et al. (2001) reported intracellular granular antigens by immunohistochemistry, which may represent lysated parasites [31] supporting the possibility of residual DNA in tissues. Similarly, Martins et al. (2008), considered parasitological cure despite the presence of residual DNA in tissues.
Cure rates described in the literature are very variable depending on *T. cruzi* strain or mouse model as well as the particular cure criteria used in each study. Discordant information was found in this area concerning DTU TcVI. C3H male mice infected with RA strain and treated with BZ for 20 days resulted in 50% of parasitological cure [32], while studies employing Tulahuen strain reached cure rates from none to 100% [13,33] suggesting that the correlation between DTU and drug sensitivity is not particularly strong. Similarly to our results, BZ treatment reduced the parasitemia and other associated parameters without achieving parasitological cure in any of the mice infected with isolates obtained from pediatric patients from Jequitinhonha Valley (Minas Gerais, Brazil) [23]. Andrade et al. (1985) described NFX sensitivity of numerous isolates and rates of cure determined by different methods were variable. Furthermore, treatment with NFX for 10 days reduced parasitemia levels below the microscopic detection limit, but failed to achieve parasitic cure assessed by qPCR in animals infected with Tulahuen or Y strains [13]. Unlike BZ, the therapeutic response and parasitological cure obtained in murine models treated with NFX have not been extensively studied. The different rates in parasitological cure obtained in experimental studies can be partly explained by the wide variety of animal models and how new biotechnological tools have modified the cure criteria. At the moment, there is no consensus on what techniques can establish unequivocally the parasitic sterilization and possibly a set of procedures (immunosuppression, qPCR, biomarkers) may provide an overall scenario to determine parasitological cure and a better success of animal model predictability.

5. Conclusion
We have studied in depth the biological and molecular properties of the VD strain, a *T. cruzi* isolate obtained from a paediatric patient with congenital Chagas disease. We conclude that the VD strain can be considered one of the representative *T. cruzi* strain, with suitable characteristics for inclusion in a regional panel of *T. cruzi* reference strains for *in vitro* and *in vivo* drug screening, among other experimental uses.

6. Acknowledgements

We would like to thank Dr. Marta Victoria Cardinal (Instituto de Ecología, Genética y Evolución de Buenos Aires - CONICET) for the useful help with the lineage identification, Juan Carlos Ramírez Gómez (Instituto de Investigaciones en Ingeniería Genética y Biología Molecular "Dr. Héctor N. Torres" – CONICET) who gently allowed the use of Nanodrop® equipment and to Dr. Héctor Freilij for the critical review of the manuscript.

7. Conflict of Interests

No conflict of interests exists in the results being presented in this paper.

8. References


**Table 1.** *T. cruzi* VD strain *in vitro* susceptibility to reference drugs (mean [95% confidence interval]) and comparison with other standard strain from same DTU.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trypomastigote LC₅₀</th>
<th>Amastigote IC₅₀</th>
<th>Trypomastigote LC₅₀</th>
<th>Amastigote IC₅₀</th>
<th>Trypomastigote LC₅₀</th>
<th>Amastigote IC₅₀</th>
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<tr>
<td>NFX</td>
<td>2.88 µM [2.31-3.60]</td>
<td>0.66 µM [0.28-1.57]</td>
<td>N/A</td>
<td>0.35 µM *</td>
<td>N/A</td>
<td>0.24 µM *</td>
</tr>
<tr>
<td>BZ</td>
<td>6.19 µM [3.86-9.92]</td>
<td>0.24 µM [0.16-0.34]</td>
<td>N/A</td>
<td>1.6 µM *</td>
<td>30.26 µM #</td>
<td>0.63 µM *</td>
</tr>
</tbody>
</table>

* [34]  # [37]  
N/A: not available.
Table 2. Parasitemia parameters evaluated in non-treated (NT), benznidazole (BZ)- or nifurtimox (NFX)-treated mice during acute phase of VD strain *T. cruzi* infection. Values are expressed in medians (range).

<table>
<thead>
<tr>
<th></th>
<th>PPP (days)</th>
<th>PP (days)</th>
<th>Day of maximum parasitemia (days)</th>
<th>Parasitemia peak (trypomastigotes/mL)</th>
<th>Parasitemia reduction from peak (%)</th>
<th>Parasitemia clearance (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>7 (5; 9) a</td>
<td>11 (8; 27) a</td>
<td>18 (14; 34) a</td>
<td>1.48 x 10^6 (0.26 x 10^6; 3.06 x 10^6) a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BZ</td>
<td>7 (7; 9) a</td>
<td>3 (1; 8) b</td>
<td>9 (7; 12) b</td>
<td>0.75 x 10^5 (0.125 x 10^5; 3.62 x 10^5) b</td>
<td>88.37</td>
<td>3 (2; 3) a</td>
</tr>
<tr>
<td>NFX</td>
<td>8 (7; 9) a</td>
<td>1 (1; 14) b</td>
<td>8 (8; 9) b</td>
<td>0.375 x 10^5 (0.125 x 10^5; 2.25 x 10^5) b</td>
<td>93.48</td>
<td>3 (3; 14) b</td>
</tr>
</tbody>
</table>

n= 17 animals per experimental group.

PPP= pre-patent period (i.e. first day of parasitemia detection).

PP= patent period.

^1 Median (range) number of doses required to induce negative parasitemia.

Values with different letters in same column are significantly different (Kruskall-Wallis; p<0.05).*
Table 3. Parasite loads and efficacy of treatment with BZ 100 mg/kg or NFX 100 mg/kg for 20 days in a murine model of acute VD strain *Trypanosoma cruzi* infection without immunosuppression, compared to untreated (NT) animals.

<table>
<thead>
<tr>
<th></th>
<th>Positive FBE (%)</th>
<th>Number of animals with a positive qPCR (%)</th>
<th>Positive mice by any method (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Skeletal muscle</td>
<td>Heart</td>
</tr>
<tr>
<td>NT</td>
<td>16 / 17 (94%)</td>
<td>17 / 17 (100%)</td>
<td>17 / 17 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BZ</td>
<td>0 / 5 (0%)</td>
<td>2 / 5 (40%)</td>
<td>1 / 5 (20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFX</td>
<td>1 / 5 (20%)</td>
<td>5 / 5 (100%)</td>
<td>4 / 5 (80%)</td>
</tr>
</tbody>
</table>

FBE=fresh blood direct examination at the end of the therapy
**Table 4.** Quantitative PCR results and efficacy of 20 days treatment with benznidazole (BZ) 100 mg/kg or nifurtimox (NFX) 100 mg/kg in a murine model of acute *T. cruzi* VD strain infection before and after immunosuppression.

<table>
<thead>
<tr>
<th></th>
<th>Positive FBE (%)</th>
<th>Blood (before CYP)</th>
<th>Blood (after CYP)</th>
<th>Skeletal muscle</th>
<th>Heart</th>
<th>Brain</th>
<th>Positive mice by any method (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 / 1 (100%)</td>
<td>1 / 1 (100%)</td>
<td>1 / 1 (100%)</td>
<td>1 / 1 (100%)</td>
<td>1 / 1 (100%)</td>
<td>1 / 1 (100%)</td>
<td>1 / 1 (100%)</td>
</tr>
<tr>
<td><strong>BZ</strong></td>
<td>8 / 12 (67%)</td>
<td>8 / 12 (67%)</td>
<td>8 / 12 (67%)</td>
<td>7 / 12 (58%)</td>
<td>7 / 12 (58%)</td>
<td>7 / 12 (58%)</td>
<td>8 / 12 (67%)</td>
</tr>
<tr>
<td><strong>NFX</strong></td>
<td>6 / 12 (50%)</td>
<td>8 / 12 (67%)</td>
<td>6 / 12 (50%)</td>
<td>7^a / 12 (58%)</td>
<td>8^a / 12 (67%)</td>
<td>6 / 12 (50%)</td>
<td>6 / 12 (50%)</td>
</tr>
</tbody>
</table>

FBE= fresh blood examination at the end of the therapy
CYP= cyclophosphamide (200 mg/kg; ip).

^a^ includes positive but not quantifiable samples.
**Figure 1.** A) Parasitemia curve during acute phase of infection with *T. cruzi* VD strain. The asterisks indicate timepoints where parasitemia levels were significantly higher in the NT group (Kruskall-Wallis; p<0.001). B) Cumulative survival curves of mice infected with *T. cruzi* throughout the acute stage. Cumulative mortality 50% (CM50) was reached at 19 dpi in NT group.
Figure 2. A) Inflammatory infiltrate scores in skeletal muscle. B) Inflammatory infiltrate scores in cardiac muscle. C) Liver morphometric analysis: number of inflammatory foci / 100 microscope fields (x400). D) Mean inflammatory infiltrate area in liver (expressed in µm²). Bars with distinct letters are significantly different (Kruskall-Wallis; p<0.05).
Figure 3. Estimated parasite load in *T. cruzi* VD strain infected, non-immunosuppressed animals after treatment. *n*: NT=17; NFX= 5; BZ=5.
Highlights

- There is lack of information on vertically transmitted *T. cruzi* strains.
- *T. cruzi* VD strain was isolated from a congenital Chagas disease patient.
- Main molecular and biological characteristics were described.
- VD strain has suitable characteristics to include in a panel of reference strains.