Accepted Manuscript

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

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Please cite this article as: Nicolás Gulin, Juliá.Ernesto., Bisio, M., Rocco, D.M., Altcheh, J., Solana, Marí.Elisa., García-Bournissen, F., Molecular and biological characterization of a highly pathogenic *Trypanosoma cruzi* strain isolated from a patient with congenital infection, *Experimental Parasitology* (2018), doi: 10.1016/j.exppara.2018.02.002.

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
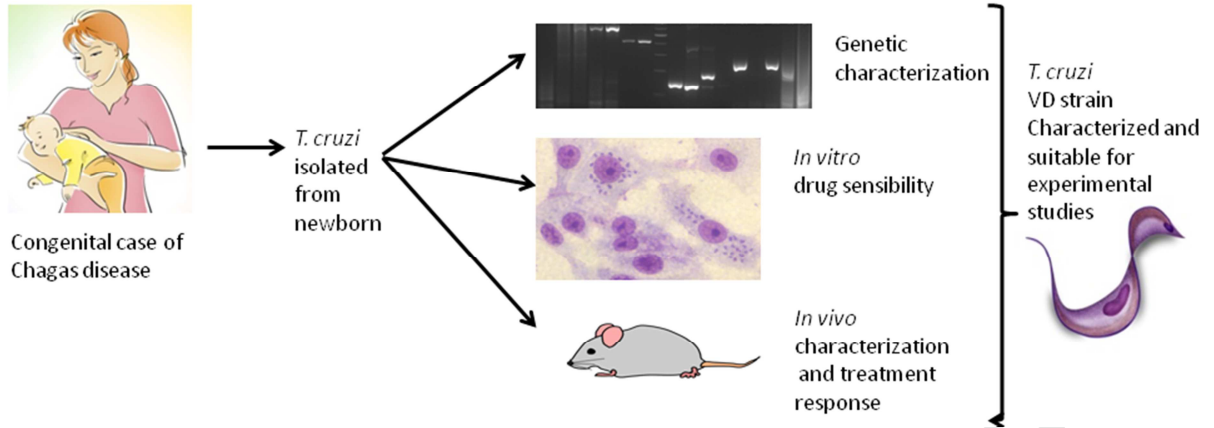
1 Running title: *T. cruzi* VD strain characterization

2

3 **Molecular and biological characterization of a highly pathogenic *Trypanosoma cruzi* strain**

4 **isolated from a patient with congenital infection**

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17

18 **Abstract**

19

20 Although many *Trypanosoma cruzi* (*T. cruzi*) strains isolated from a wide range of hosts have

21 been characterized, there is a lack of information about biological features from vertically

22 transmitted strains.

23 We describe the molecular and biological characteristics of the *T. cruz*i VD strain isolated

24 from a congenital Chagas disease patient.

25 The VD strain was typified as DTU TcVI; *in vitro* sensitivity to nifurtimox (NFX) and

26 beznidazole (BZ) were 2.88 μM and 6.19 μM respectively, while inhibitory concentrations for

27 intracellular amastigotes were 0.24 μM for BZ, and 0.66 μM for NFX. Biological behavior of

28 VD strain was studied in a mouse model of acute infection, resulting in high levels of

29 parasitemia and mortality with a rapid clearence of bloodstream trypomastigotes when

30 treated with BZ or NFX, preventing mortality and reducing parasitic load and intensity of

31 inflammatory infiltrate in skeletal and cardiac muscle. Treatment-induced parasitological

32 cure, evaluated after immunossupression were 41% and 35% for BZ and NFX treatment

33 respectively, suggesting a partial response to these drugs in elimination of parasite burden.

34 This exhaustive characterization of this *T. cruzi* strain provides the basis for inclusion of this

35 strain in a panel of reference strains for drug screening and adds a new valuable tool for the

36 study of experimental *T. cruzi* infection.

37 **Keywords**

38 *Trypanosoma cruzi;* Chagas' disease; Congenital transmission; Discrete Typing Unit

39 Benznidazole; Nifurtimox.

40

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44

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 propierties 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

74 studies, there is a lack of information on biological behaviour, drug sensitivity and treatment

75 response for *T. cruzi* strains isolated from transplacental infection.

76 The aim of this work was to characterize the biological and molecular properties of a *T. cruzi*

77 isolate, the VD strain, obtained from a pediatric congenital case.

78

79 **2. Materials and Methods**

80

81 2.1 Clinical case

82

83 A three months old baby born in a non-endemic area to a previously non treated mother

84 with chronic indeterminate phase of Chagas disease, was referred to the Service of

85 Parasitology and Chagas Disease, "Dr. Ricardo Gutiérrez" Children's Hospital (Buenos Aires,

86 Argentina) with low weight, jaundice, hepatosplenomegaly and positive *T. cruzi* parasitemia.

87 The child was treated initially with BZ but interrupted due to gastric intolerance; treatment

88 was changed to NFX with good tolerance and clinical response. After treatment

89 completition, the patient returned to his hometown and was lost to follow up.

90

91 2.2 Strain isolation

92

93 A blood sample from the pediatric patient was inoculated subcutaneously in 14 days old

94 Rockland mice (n=6) (González-Cappa, SM, laboratory records). Parasites obtained from this

95 initial isolate have been maintained by weekly passages in 21 days old CF-1 male mice,

96 infected with 1×10^5 bloodstream trypomastigotes by intraperitoneal (ip) route. Bloodstream

97 trypomastigotes are also routinely stored inliquid nitrogen. This *T. cruzi* isolate wil be

98 referred to as VD strain. For experimental purposes, VD strain bloodstream trypomastigotes

99 were purified from heparinized mice blood as previously described [10].

100

101

102

103

104   2.3 Compounds and reagents

105

106   For *in vitro* assays, pure powder of BZ (N-Benzyl-2-nitroimidazole acetamide) (provided by

107   Elea®, Buenos Aires, Argentina) and NFX (3-methyl-4- (5'-nitrofurfurylideneamina)

108   tetrahydro-4H-thiazine -1,1-dioxide) (provided by Bayer®, Germany) were suspended in

109   dimethylsulfoxide (DMSO). For *in vivo* assays, BZ (Abarax®, Elea) and NFX (Lampit®, Bayer)

110   tablets were pulverized and resuspended in a 0.25% carboxymethylcellulose (CMC) solution

111   (Sigma-Aldrich, USA).

112

113   2.4 *In vitro* assays

114

115   2.4.1 Cell culture

116

117   Vero cells were grown in 25 cm^2 tissue-culture flasks , using L-glutamine RPMI-1640 medium

118   supplemented with 5% of FCS, 10 mM HEPES, 100 UI/mL penicillin and 50 µg/mL

119   streptomycin, and incubated in 5% CO_2 atmosphere at 37°C.

120

121   2.4.2 Trypomastigotes drug susceptibility

122

123   VD strain trypomastigotes purified from mice blood were seeded in 96-wells polystyrene flat

124   bottom plates in 100 µL of RPMI-5% FCS ($1x10^6$ parasites/mL), exposed to five serial dilutions

125   of NFX (100-0.3 µM) or BZ (230-0.1 µM) and incubated at 37°C in a 5% CO_2–95% air mixture

126   for 24 hours. Motile parasites were counted in a Neubauer chamber. Each drug

127   concentration was evaluated by triplicate and compared to untreated control cultures. The

128   50% lytic concentrations (LC_{50}), defined as the drug concentration that resulted in a 50%

129   reduction of trypomastigotes viability compared to the non-treated controls, was estimated

130   by non-linear regression analysis [11].

131

132

133

134   2.4.3 Amastigote growth inhibition assay

135

136   Cesium 137 irradiated Vero cells (2000 rad) were plated overnight at 37°C-5% CO_2 to a

137   density of $5x10^4$ of cells/well, in 24-well plates with rounded coverslips at the bottom.

138   Infection conditions were previously optimized with $2,5x10^5$ parasites/well and incubated for

139   3 hours at 37°C. Then,wells were washed with PBS to remove non-attached parasites and

140   fresh RPMI media containing NFX (20 to 0.2 µM), BZ (10 to 0.16 µM) or medium was added.

141   Drug-contraining media was renewed every 24 hours for 3 consecutive days.; Then, cells

142   were washed with PBS, fixed with methanol and stained with Giemsa. Each drug

143   concentration was evaluated by triplicate. The 50% inhibitory concentration (IC_{50}), defined

144   as the drug concentration required to achieve 50% inhibition of intracellular amastigote

145   growth by counting number of amastigotes per cell, was determined by non-linearregression

146   analysis [11].

147

148   2.5 *In vivo* assays

149   2.5.1 Animal breeding conditions

150   Twenty days old female BALB/c mice (15 ± 2 grams) were obtained from the Animal Facilities

151   at the Faculty of Veterinary Sciences, University of Buenos Aires (Argentina) and maintained

152   under conventional closed barriers at the "Dr. Ricardo Gutiérrez" Children's Hospital Animal

153   Facilities. Animals were housed in 600 cm^2 polycarbonate at 4-5 animals per cage. Mice were

154   individually identified and cages were properly labelled. Cages were filled with irradiated

155   chip-bedding and changed once a week. Mice had access to food and water *ad libitum*.

156   Macroenvironmental conditions included a 12:12 hs light:dark cycle (starting at 6 a.m.),

157   controlled temperature of 20 to 22 °C, and 45% to 55% humidity.

158

159

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162

163 2.5.2 Infection and treatment

164

165 Mice were infected at 5 to 7 weeks of age (16.25 ± 2.09 grams) by the intraperitoneal route

166 (ip) with 500 bloodstream VD strain trypomastigotes. Blood direct microscopic observation

167 was performed starting at the 5th day post infection (dpi). Treatment was begun when

168 parasites were detected in circulation; animals were then randomly assigned to the

169 following groups: BZ (n = 17), NFX (n = 17) or infected non-treated (NT, n = 17).

170 Treatment was administered orally for 20 consecutive days at a dose of 100 mg/kg/day for

171 NFX or BZ, using disposable tips and an automatic pipette, in a final volume of 50 µL per

172 animal per dose. The doses, length and route of administration were chosen based on

173 previous published literature [12,13].

174 Parasitemia was evaluated three times a week during therapy by pricking the tail and

175 collecting 5 µl of fresh blood. Blood was incubated with red blood cell lysis solution (20 µl of

176 Tris-buffered 0.83% ammonium chloride pH 7.2), and motile parasites were counted in a

177 Neubauer chamber. In addition, the following parasitemia parameters were recorded:

178 prepatent period (defined as time between infection and detection of circulating parasites),

179 patent period (time elapsed between parasitemia onset and negativization), parasitemia

180 peak (highest parasitemia level), and number of drug doses needed to turn parasitemia

181 undetectable. Animals were periodically weighed to adjust doses and to assess changes in

182 body weight. Body temperature was determined rectally with a pediatric digital

183 thermometer. Mortality was recorded daily. To avoid unnecessary pain and stress, pre-

184 established anticipated endpoints were used, and animals were euthanized if they fullfiled

185 any of these criteria (i.e. 20% weight loss from initial body weight, body temperature lower

186 than 33.5°C, or parasitemia higher than $2x10^6$ trypomastigotes/mL). Euthanasia was

187 performed with CO_2 inhalation in a saturated chamber or sodium pentothal overdose (300

188 mg/kg, ip).

189

190

191   2.5.3 Determination of parasitological cure

192

193   At the end of the treatment, 5 surviving animals from each group were euthanized, and

194   blood and tissue samples were obtained for *T. cruzi* DNA detection by real-time PCR (qPCR)

195   and histopathology. Remaining animals with undetectable parasitemia were left without

196   treatment for 10 days, with periodic checks for re-emergence of bloodstream parasites, and

197   then subjected to a cyclophosphamide (CYP)-based immunosuppression protocol to rule out

198   parasite reemergence from sanctuary sites in tissues [14]. Briefly, CYP was given once a

199   week by ip injection at 200 mg/kg for a total of 4 administrations. Animals were euthanized

200   if parasite ressurgence was observed or, if not, after four cycles of immunosuppression,

201   tissues (heart, skeletal muscle and brain) and blood were collected for confirmation of cure

202   by histopathology and qPCR.

203

204   2.6 Tissue sample preparation and histological evaluation

205

206   Samples from heart, skeletal muscle, brain, liver, kidney, colon and uterus were collected

207   from all animals to evaluate inflammation and presence of amastigote nests. Samples were

208   fixed in buffered 10% formaldehyde, dehydrated, paraffin-embedded, and 5 μm thick

209   sections were stained with haematoxylin and eosin (H&E).

210   Evaluation of the tissue specimens was performed by light microscopy by a researcher

211   blinded to the treatment assignment. Presence of amastigote nests was recorded and the

212   degree of myocardial and skeletal inflammation was scored as described previously [15,16].

213   General histoarchitecture, inflammatory infiltrates, degree of vascular congestion and

214   presence of amastigote nests were evaluated in liver, kidney, colon, uterus and brain.

215   Number of inflammatory foci was determined in 100 fields at 400X magnification and extent

216   of infiltration (expressed in μm^2) was measured using Infinity Analyse program 6.5.0

217   (Lumenera Corp®).

218

219   2.7 Quantitative PCR (qPCR)

220

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

227   *T. cruzi* DNA amplification was performed using *cruzi 1* [5'-3'-ASTCGGCTGATCGTTTTCGA] and
228   *cruzi 2* [5'-3'-AATTCCTCCAAGCAGCGGATA] primers, which amplify a 166-bp specific
229   fragment, corresponding to satellite DNA. The detection was performed using TaqMan®
230   probe *cruzi 3* [5'-3'CACACACTGGACACCAA] as previously described [17]. The standard curve
231   allowed quantification of DNA between a range of $1.6x\ 10^{0}$ to $8x10^{5}$ parasite
232   equivalents/mL. An internal amplification standard (IAC) of DNA extraction (2 ng) was
233   included in each GEB sample.

234

235   2.8 Discrete Typing Unit (DTU) determination

236

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

243

244

245

246

247 2.9 Statistical Analysis

248

249 For *in vivo* studies, data was subjected to analysis of variance test (ANOVA) with post-hoc

250 correction for multiple comparisons with Bonferroni test or non-parametric Kruskal-Wallis

251 test, and compared in pairs depending on data distribution. Survival analysis was performed

252 using Kaplan-Meier test. In all cases, p-values<0.05 were considered statistically significant.

253 Statistical analyses for *in vivo* assays were performed with InfoStat/P 2014 (Universidad

254 Nacional de Córdoba, Argentina). BZ and NFX LC_{50} and IC_{50} were determined by non-linear

255 regression using GraphPad Prism 5.03 software (GraphPad Software, Inc. USA).

256 Graphics were prepared with GraphPad Prism 5.03. Values in tables and graphs are

257 expressed in mean values with standard deviation unless otherwise indicated.

258

259 2.10 Ethical statement

260 Animals were acclimatized to new housing conditions and habituated to routine handling by

261 trained personnel for two weeks prior to the experiment and procedures were performed

262 according to local guidelines [19]. Protocol was approved by the Institutional of Animal Care

263 and Use Committee from Faculty of Veterinary – University of Buenos Aires (# Protocol:

264 2014/4). Anticipated endpoints (see above) were impremented to avoid unnecessary

265 suffering.

266

267 **3. Results**

268

269 3.1 Discrete Typing Unit (DTU) determination

270

271 DNA analysis from cell-culture derived VD strain trypomastigotes revealed that it belongs to

272 the DTU TcVI according to SL-PCR II 425 bp, heminested 24αS ribosomal PCR 140 bp and A10

273 fragment 630 bp (Supplementary Material, SM 1).

274

275

276   3.2 *In vitro* drug sensitivity

277

278   VD strain susceptibility to reference drugs (i.e. BZ and NFX) both in trypomastigotes (LC_{50})

279   and intracellular amastigotes (IC_{50}) is shown in Table 1 and dose-response curves are

280   displayed in Supplementary Material 2 (SM2).

281

282   3.3 Course of acute infection with *T. cruzi* VD strain in a murine model

283

284   Parasites were detected in mice blood at 8 (range 5 to 9) days post infection (dpi) (i.e. afterip

285   innoculation), at which point they were divided into treatment (NFX or BZ) or NT groups.

286   Mice from the NT group developed high levels of bloodstream VD trypomastigotes (Figure

287   1A), unlike BZ or NFX groups which showed treatment response with a rapid decrease in

288   bloodstream parasites. Parasitemia parameters are shown in Table 2.

289   BZ and NFX were well tolerated throughout the study by all treated mice. Animals treated

290   with BZ or NFX were clinically healthy, with shiny and well groomed fur, showing alert

291   behavior, connected with environment. In contrast, animals in the NT group failed to recover

292   clinically, showing a soiled and bristly coat, with hunched guarding posture and partially

293   closed eyelids accompanied by tearing. Reduced mobility with lethargic reflexes and

294   decreased awareness were also observed. These clinical signs were accompanied by weight

295   loss and decreased body temperature, reaching the maximum pre-established tolerable

296   endpoint near peak parasitemia, at approximately 21 dpi (Figure 1B).

297   None of the animals in the NT group gained body weight during follow up, reaching weight

298   nadir at 21 dpi. On the other hand, animals treated with BZ or NFX exhibited a less

299   pronounced weight loss, especially in the NFX group, and a rapid weight recovery during the

300   treatment period. Moreover, animals from the NT group exhibited lower average body

301   temperature compared to NFX or BZ treated animals, starting from 12 dpi and reaching

302   values closely to hypothermia at approximately 21 dpi, coinciding with peak parasitemia

303   levels (Supplementary Material SM3).

304

305

306   3.4 Histopathological features

307

308   The skeletal muscle in all studied animals in the NT group showed a high degree of

309   rhabdomyolysis with mononuclear inflammatory infiltrates giveng a mean inflamatory score

310   of 3.5 over 5 and muscle fiber calcifications were observed in 4 samples. Amastigote nests

311   could be observed in 82.35% of animals (14 / 17). By contrast, animals treated with BZ or

312   NFX exhibited comparatively lower degrees of inflammation, with scores ranging from 0.5 to

313   3 (over 5), and mild to moderate interfibrillar mononuclear inflammatory infiltrates without

314   rhabdomyolysis (Figure 2A). Amastigote nests were observed in 4 of 17 samples from BZ

315   group, whilst specimens from NFX-treated animals had no detectable amastigote nests.

316   Animals from the NT group also showed myocardial inflammatory foci, in some cases with

317   local extension without involving complete heart wall (inflammation score=3) with

318   predominantly mononuclear cells infiltrates. Inflammation scores ranged from 1 to 3 (over

319   5). Also, 65% (11 / 17) of NT mice showed amastigote nests within cardiac fibers. BZ-treated

320   animals showed greater variability in degree of inflammation, with scores ranging from 0 to

321   4, with focal, but not confluent, infiltrates in the peripheral heart wall being the

322   predominant finding. Only 29.5% (5/17) BZ-treated animals exhibited amastigote nests in

323   myocardial samples. Mice from the NFX group showed myocardial inflammation ranging

324   from 0 to 3 points (over 5). Similarly to BZ-treated animals, focal lesions with mononuclear

325   infiltrates were predominant. However, amastigote nests were observed only in one animal

326   (6%; 1 / 17). (Figure 2B)

327   Focal mononuclear infiltrates were also the main feature in liver samples from all

328   experimental groups. No differences were observed among groups in the number of

329   inflammatory foci, but their sizes were larger in NT animals, followed by those treated with

330   BZ (Figures 2C-2D). Amastigote nests were not identified in liver histological sections from

331   any experimental group.

332   No significant morphological and/or histological alterations, nor amastigote nests were

333   observed in kidney, colon, uterus and brain samples of any experimental group.

334

335

336   3.5 Assesment of treatment-induced parasitological cure

337

338   After completion of 20 consecutive days of therapy with either BZ or NFX, 5 animals from

339   each group were euthanized to obtain blood samples and target organs (skeletal muscle,

340   heart and brain) in order to analyze parasite burden by qPCR. Table 3 shows percentage of

341   animals with parasitic esterilization (without immunosupression) in each treatment group

342   (i.e. BZ or NFX). Estimated parasitic loads are illustrated in Figure 3. Parasitological cure rates

343   in BZ or NFX treated animals after inmunosuppression with CYP are shown in Table 4.

344   In summary, adding all negative animals irrespective of whether they received

345   immunossupression or not, the overall parasitological cure was observed in 41% (7/17) of

346   the BZ-treated animals, and in 35% (6/17) of the NFX-treated animals.

347

348   **4. Discussion**

349

350   We have conducted the first exhaustive description of the main biological and molecular

351   characteristics of the *T. cruzi* VD strain, originally isolated by our group from a congenital

352   Chagas disease case.

353   Based on well-established DTU methods, the VD strain belongs to DTU TcVI, supporting

354   previous findings [20] and suggesting genetic stability through mice passages. DTU TcVI is

355   one of the most prevalent *T. cruzi* genotype in domestic trasmission cycle and it have been

356   linked to congenital cases [18].

357   *In vitro* susceptibility to BZ and NFX was assesed both in bloodstream trypomastigotes and

358   amastigotes. Inhibitory concentration values for BZ and NFZ were similar to those reported

359   from *T. cruzi* reference strains within DTU Tc VI (Tulahen and CL-Brener), but considerably

360   below the lytic concentration from Tulahuen trypomastigotes (Table 1). Unfortunately,

361   comparison with NFX *in vitro* sensitivity on *T. cruzi* trypomastigotes was hampered because

362   most LC_{50} were established on epimastigote stage, which does not represent the same

363   biological and clinical characteristics of the trypomastigote and amastigote stages, incuding

364   drug sensitivity to reference drugs.

365 Given the extreme variability in drug sensitivity even within the same DTU, there is no
366 convincing evidence yet to support the association between *in vitro* sensitivity to
367 conventional trypanocidal drugs and phylogenetic features. There are also no consistent
368 data to support any correlation between DTU and morbidity, risk of reactivation or
369 congenital transmission in humans [3].

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

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

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

391 In our model, parasitemia reached maximum values by 18 dpi in NT animals, accompanied
392 by symptoms compatible with systemic infection and reduced weight and body
393 temperature. Weight loss is typical of the experimental acute phase and could be related to
394 the lower average water and food consumption.

395   Histopathological findings during the acute phase of infection showed a marked tropism for

396   skeletal and cardiac muscle. NT animals were significantly more likely to have amastigotes

397   nests observed in these tissues than those treated with BZ and NFX, even in those who

398   underwent immunosuppression. These results contrast with observations from models with

399   CL-Brener or RA strains (both DTU TcVI) with a marked tissue pantropism [25,26].

400   Morphometric analysis of liver samples showed larger inflammatory areas in NT animals

401   compared to those treated with conventional drugs. However, treatment with BZ and NFX

402   was unable to decrease concomitant liver inflammation. Future studies should focus on

403   defining whether this infiltrate is a consequence of parasite infiltration, the toxic action of

404   nitroheterocyclic drugs or due a combined effect between *T. cruzi* infection and treatment,

405   as suggested previously [27].

406   Absence of amastigote nests in liver agrees with previous data from isolates from *T.*

407   *rubrovaria* (DTU TcIII) in female Swiss mice [28] and might be related to the involvement of

408   this organ as a major site of immunological elimination of parasites [29].

409   An immunosuppression protocol with CYP and and qPCR quantification of parasite load in

410   blood and target tissues were chosen to determine parasitological cure.   The

411   immunosuppression protocol proved to be a good marker for treatment failure, as all

412   animals showing parasite DNA in at least one of the analyzed tissues subsequently

413   reactivated after administration of CYP.

414   Importantly, none of the BZ treated animals showed persistent parasitemia by fresh blood

415   examination after end of therapy, while only one mouse treated with NFX showed blood

416   trypomastigotes after 20 consecutive days of treatment. Two heart and skeletal muscle

417   samples from NFX-treated animal which did not reactivate after immunosuppresion were

418   positive by qPCR, but due to their low parasitic load could not be quantified. Interestingly,

419   DNA was not detectable by qPCR in blood of these mice. The presence of residual DNA in

420   tissues or possible PCR cross contamination could explain these non quantifiable positive

421   levels of *T. cruzi* DNA in tissues. Guarner et al. (2001) reported intracellular granular

422   antigens by immunohistochemistry, which may represent lysated parasites [31] supporting

423   the possibility of residual DNA in tissues. Similarly, Martins et al. (2008), considered

424   parasitological cure despite the presence of residual DNA in tissues.

425 Cure rates described in the literature are very variable depending on *T. cruzi* strain or mouse

426 model as well as the particular cure criteria used in each study- Discordant information was

427 found in this area concerning DTU TcVI. C3H male mice infected with RA strain and treated

428 with BZ for 20 days resulted in 50% of parasitological cure [32], while studies employing

429 Tulahuen strain reached cure rates from none to 100% [13,33] suggesting that the

430 correlation between DTU and drug sensitivity is not particularly strong.

431 Similarly to our results, BZ treatment reduced the parasitemia and other associated

432 parameters without achieving parasitological cure in any of the mice infected with isolates

433 obtained from pediatric patients from Jequitinhonha Valley (Minas Gerais, Brazil) [23].

434 Andrade et al. (1985) described NFX sensitivity of numerous isolates and rates of cure

435 determined by different methods were variable. Furthermore, treatment with NFX for 10

436 days reduced parasitemia levels below the microscopic detection limit, but failed to achieve

437 parasitic cure assesed by qPCR in animals infected with Tulahuen or Y strains [13]. Unlike BZ,

438 the therapeutic response and parasitological cure obtained in murine models treated with

439 NFX have not been extensively studied.

440 The different rates in parasitological cure obtained in experimental studies can be partly

441 explained by the wide variety of animal models and how new biotechnological tools have

442 modified the cure criteria. At the moment, there is no consensus on what techniques can

443 establish unequivocally the parasitic sterilization and possibly a set of procedures

444 (immunosuppression, qPCR, biomarkers) may provide an overall scenary to determine

445 parasitological cure and a better success of animal model predictability.

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453 **5. Conclusion**

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

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461 **6. Acknowledgements**

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

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469 **7. Conflict of Interests**

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

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481 **8. References**

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

Drug	VD		CL-Brener		Tulahuen	
	Trypomastigote LC_{50}	Amastigote IC_{50}	Trypomastigote LC_{50}	Amastigote IC_{50}	Trypomastigote LC_{50}	Amastigote IC_{50}
NFX	2.88 µM [2.31-3.60]	0.66 µM [0.28-1.57]	N/A	0.35 µM *	N/A	0.24 µM *
BZ	6.19 µM [3.86-9.92]	0.24 µM [0.16-0.34]	N/A	1.6 µM*	30.26 µM [#]	0.63 µM*

* [34]

[37]

N/A: not available.

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610   **Table 2.** Parasitemia parameters evaluated in non-treated (NT), benznidazole (BZ)- or

611   nifurtimox (NFX)-treated mice during acute phase of VD strain *T. cruzi* infection. Values are

612   expressed in medians (range).

	PPP (days)	PP (days)	Day of maximum parasitemia (days)	Parasitemia peak (trypomastigotes/mL)	Parasitemia reduction from peak (%)	Parasitemia clearence (days) [1]
NT	7 (5; 9) a	11 (8; 27) a	18 (14; 34) a	1.48×10^6 (0.26×10^6; 3.06×10^6) a	-	-
BZ	7 (7; 9) a	3 (1; 8) b	9 (7; 12) b	0.75×10^5 (0.125×10^5; 3.62×10^5) b	88.37	3 (2; 3) a
NFX	8 (7; 9) a	1 (1; 14) b	8 (8; 9) b	0.375×10^5 (0.125×10^5; 2.25×10^5) b	93.48	3 (3; 14) b

613   n= 17 animals per experimental group.
614   PPP= pre-patent period (i.e. first day of parasitemia detection).
615   PP= patent period.
616   [1] Median (range) number of doses required to induce negative parasitemia.
617   Values with different letters in same column are significantly different (Kruskall-Wallis;
618   p<0,05).*

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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 immunosuppresion, compared to untreated (NT) animals.

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

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.

		Number of animals with a positive qPCR (%)					
	Positive FBE (%)	Blood (before CYP)	Blood (after CYP)	Skeletal muscle	Heart	Brain	Positive mice by any method (%)
NT	1 / 1 (100%)	1 / 1 (100%)	1 / 1 (100%)	1 / 1 (100%)	1 / 1 (100%)	1 / 1 (100%)	1 / 1 (100%)
BZ	8 / 12 (67%)	8 / 12 (67%)	8 / 12 (67%)	7 / 12 (58%)	7 / 12 (58%)	7 / 12 (58%)	8 /12 (67%)
NFX	6 / 12 (50%)	8 / 12 (67%)	6 / 12 (50%)	7 [a] / 12 (58%)	8 [a] / 12 (67%)	6 / 12 (50%)	6 / 12 (50%)

FBE= fresh blood examination at the end of the therapy

CYP= cyclophosphamide (200 mg/kg; ip).

[a] includes positive but not quantifiable samples.

666 **Figure 1.** A)*. Parasitemia curve during acute phase of infection with *T. cruzi* VD strain. The

667 asterisks indicate timepoints where parasitemia levels were significantly higher in the NT

668 group (Kruskall-Wallis; p<0,001). B). Cumulative survival curves of mice infected with *T. cruzi*

669 throughout the acute stage. Cumulative mortality 50% (CM_{50}) was reached at 19 dpi in NT

670 group.

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696 **Figure 2.** A) Inflammatory infiltrate scores in skeletal muscle. B) Inflammatory infiltrate

697 scores in cardiac muscle. C) Liver morphometric analysis: number of inflammatory foci / 100

698 microscope fields (x400). D) Mean inflammatory infiltrate area in liver (expressed in μm^2).

699 Bars with distinct letters are significantly different (Kruskall-Wallis; $p<0.05$).

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725 **Figure 3.** Estimated parasite load in *T. cruzi* VD strain infected, non-immunosuppressed
726 animals after treatment. *n*: NT=17; NFX= 5; BZ=5.

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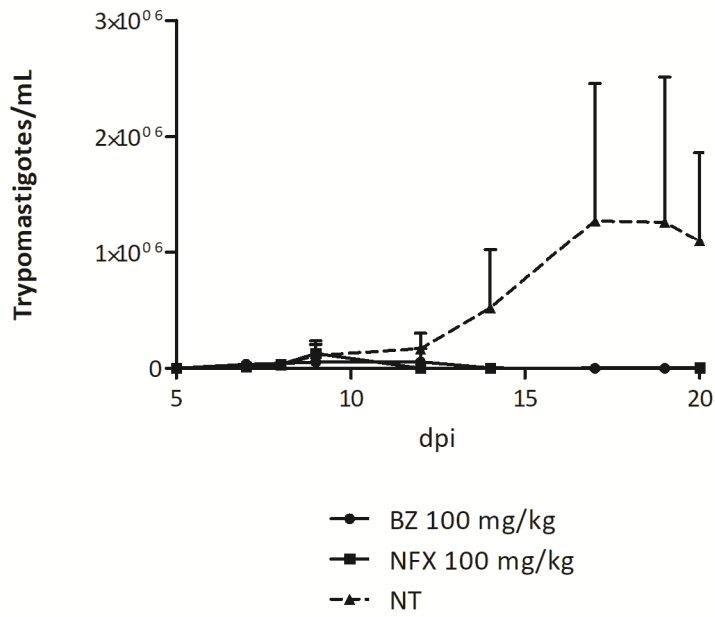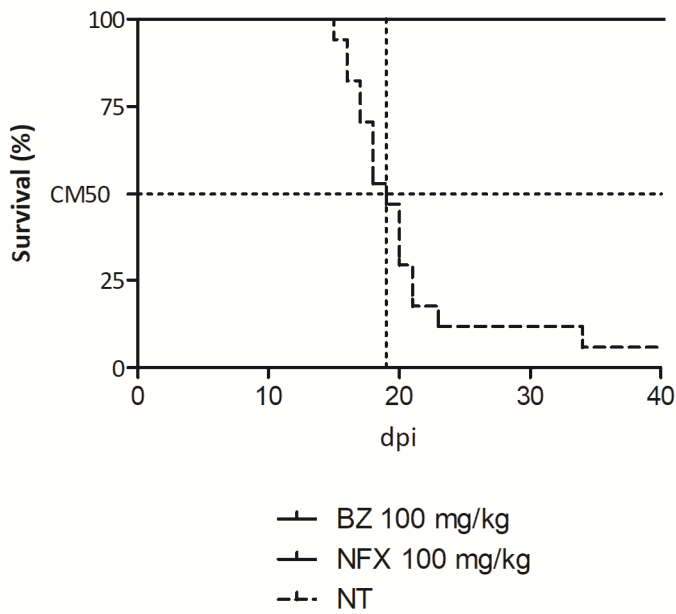
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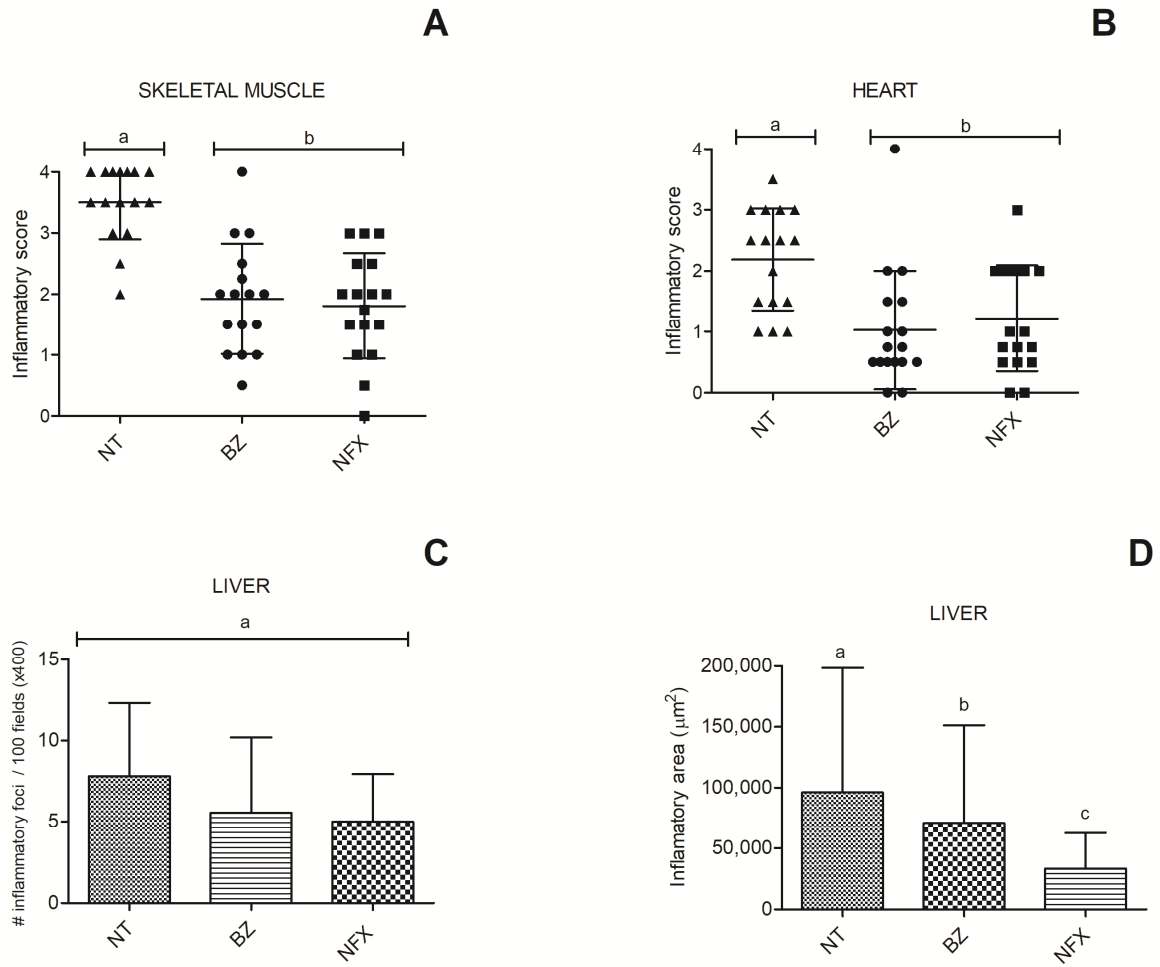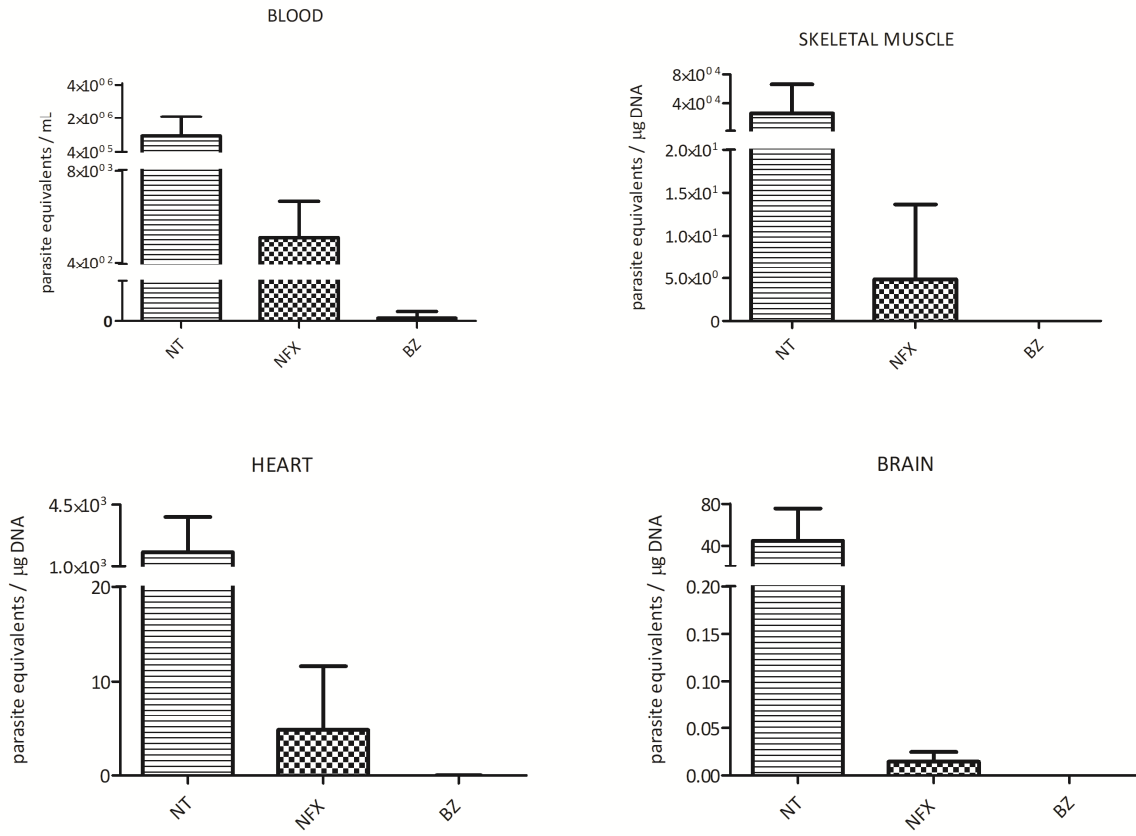
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A

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SKELETAL MUSCLE

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LIVER

BLOOD

SKELETAL MUSCLE

HEART

BRAIN

Highlights

- There is lack of information on vertically transmitted *T. cruzi* strains.
- *T. cruz*i 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.