1 **Title:** Prophylactic low-dose, bi-weekly benznidazole treatment fails to prevent *Trypanosoma cruzi*

2 infection in dogs under intense transmission pressure.

3 **Short title:** Drug prophylaxis fails to prevent *T. cruzi* infection

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14

Abstract

Trypanosoma cruzi naturally infects a wide variety of wild and domesticated mammals, in addition to humans. Depending on the infection dose and other factors, the acute infection can be life-threatening, and in all cases, the risk of chagasic heart disease is high in persistently infected hosts. Domestic, working, and semi-feral dogs in the Americas are at significant risk of *T. cruzi* and in certain settings in the southern United States, the risk of new infections can exceed 30% per year, even with the use of vector control protocols. In this study, we explored whether intermittent low-dose treatment with the trypanocidal compound benznidazole (BNZ) during the transmission season, could alter the number of new infections in dogs in an area of known, intense transmission pressure. Preliminary studies in mice suggested that twice-weekly administration of BNZ could prevent or truncate infections when parasites were delivered at the mid-point between BNZ doses. Pre-transmission season screening of 126 dogs identified 53 dogs (42.1%) as *T. cruzi* infection positive, based upon blood PCR and Luminex-based serology. Serial monitoring of the 67 uninfected dogs during the high transmission season (May to October) revealed 15 (22.4%) new infections, 6 in the untreated control group and 9 in the group receiving BNZ prophylaxis, indicating no impact of this prophylaxis regimen on the incidence of new infections. Although these studies suggest that rigorously timed and more potent dosing regimen may be needed to achieve an immediate benefit of prophylaxis, additional studies would be needed to determine if drug prophylaxis reduced disease severity despite this failure to prevent new infections.

Author Summary. *Trypanosoma cruzi*, the parasite that causes Chagas disease, circulates extensively in the southern U.S. and working dog populations in south-central Texas are at very high risk of infection, and morbidity and early mortality due to this infection. In this study, we used low level administration of an FDA-approved drug during the transmission season to attempt to prevent new infections in these dogs. Although that effort failed, the study revealed new information about the transmission dynamics and generation of antibody responses and immune control of infection in this high-transmission setting.

39 **Introduction**

40 Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a problem for human and
41 animal health across the Americas where triatomine vectors are endemic. *T. cruzi* is predominantly
42 transmitted in the feces of infected triatomines through contact with wounds or mucous membranes or
43 ingestion of infected insects or fecal material [1]. Oral transmission is thought to be the most important
44 route in domestic dogs and is a highly efficient mode of transmission [2-5].

45 Dogs are important to study in the context of Chagas disease for at least three reasons: i) they
46 experience similar disease progression to humans, so are useful to study as a model for human disease
47 especially in considering treatments; ii) they share exposure to vectors with humans in domestic and
48 peridomestic environments and therefore may serve as sentinels for human health risk [6-8]; and iii)
49 canine Chagas disease is an increasingly recognized problem in veterinary medicine, especially in the
50 southern U.S. [9], leading to dog mortality [10].

51 Large multi-dog kennels in central and south Texas have emerged as settings with particularly high
52 transmission risk, with a recent study documenting incidence of over 30 infections per 100 dogs per year
53 [11], despite variable vector control efforts in and around the kennels. Although some therapeutic
54 regimens have shown promise in reducing disease impact in dogs infected with *T. cruzi* [12], no
55 therapeutics have been evaluated as potential preventatives of *T. cruzi* infection in dogs.

56 Here we trialed a novel approach for preventing new infections in dogs within multi-dog kennel
57 environments with a history of triatomine occurrence and canine Chagas disease. Disappointingly,
58 prophylaxis during the period of peak adult vector activity [13] with benznidazole (BNZ), an FDA-
59 approved drug used in the treatment of human *T. cruzi* infection, had no impact on the incidence of new
60 infections in this setting, However the long-term impact of prophylaxis on clinical disease was not

61 monitored. Further, this study design provides a model for future evaluation of different prophylactic
62 regimens (higher dose; treatments earlier in the season) in high transmission settings.

63 **Material and Methods**

64 **Study Design**

65 For the studies in mice, female mice, 8-12 weeks old were used for infections throughout the study.
66 Sample sizes were determined based upon our knowledge of heterogeneity in parasite burden during *T.*
67 *cruzi* infection and published reports using similar experimental strategies. Data collected were included
68 if productive *T. cruzi* infection was established (visualized by luciferase imaging or flow cytometry by
69 detection of *T. cruzi*-tetramer specific $CD8^+$ T cells). The investigators were not blinded during the
70 collection or analysis of data and mice were randomly assigned to treatment groups prior to the start of
71 each experiment.

72 For studies in dogs, we formed a small network of kennels in central and south Texas with a history of
73 triatomine vector occurrence and canine Chagas disease with owners who were willing to participate in
74 the study. Kennels are identified through (i) canine patients with Chagas disease presenting to the
75 cardiology unit at the TAMU VMTH and (ii) the TAMU Kissing Bug Community Science program, in which
76 many dog owners submit triatomines collected from large kennel environments. At these large kennels,
77 dogs are primarily bred and trained to aid hunting parties. Approximately 40-80 dogs reside within each
78 kennel, and the predominant breeds include Belgian Malinois, Brittany spaniels, English pointers,
79 German shorthaired pointers, Labrador retrievers, and hound dogs. Dogs >3 months of age, including
80 males and females, were eligible for enrollment. Dogs identified after blood screening as seronegative
81 based on multiplex serology and PCR-negative were randomly assigned to treatment groups (Table S1).
82 At each sampling event, up to 6ml of blood was collected from dogs via jugular venipuncture into clot

83 activator tubes which were shipped on ice overnight to the laboratory after which serum was separated
84 and used for antibody tests, and the clot was used for DNA extractions and PCR testing.

85 The dog owners/kennel managers gave prophylaxis by placing the encapsulated BNZ in the back of the
86 dog's mouth and encouraging swallowing; in some cases, the capsules were placed into cheese or other
87 food for the dog to consume. Neither the investigators, private veterinarian providing care to the dogs
88 in the field, nor the dog owners were blinded to the treatment groups and no placebo was given to
89 control dogs.

90 **Mice, parasites and infections**

91 C57BL/6J (Stock No:000664) mice (C57BL/6 wild-type) were purchased from The Jackson Laboratory (Bar
92 Harbor, ME) and C57BL/6J-IFN-gamma knockout mice (also known as B6.129S7-Ifngtm1Ts/J; The
93 Jackson Laboratory stock No 002287) were bred in-house at the University of Georgia Animal Facility. All
94 the animals were maintained in the University of Georgia Animal Facility under specific pathogen-free
95 conditions. *T. cruzi* tissue culture trypomastigotes of the Colombiana strain co-expressing firefly
96 luciferase and tdTomato reporter proteins, generated as described previously [14, 15], were maintained
97 through passage in Vero cells (American Type Culture Collection (Manassas, VA)) cultured in RPMI 1640
98 medium with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. Mice were infected
99 intraperitoneally with 10^3 tissue culture trypomastigotes of *T. cruzi*.

100 **Drug treatment and in vivo imaging**

101 Mice were treated with benznidazole twice weekly at a 100mg/kg/day and infected midway during the
102 3rd week of treatment (Figure 1). Benznidazole (BNZ – Elea Phoenix, Buenos Aires, Argentina) was
103 prepared by pulverization of tablets followed by suspension in an aqueous solution of 1% sodium
104 carboxymethylcellulose with 0.1% Tween 80 and delivered orally by gavage at a concentration dosage of
105 100mg/kg of body weight. Each mouse received 0.2 ml of this suspension. Luciferase-expressing

106 parasites were quantified in mice by bioluminescent detection. Mice were injected intraperitoneally
107 with D-luciferin (150 mg/kg; PerkinElmer, Waltham, MA) and anesthetized using 2.5% (vol/vol) gaseous
108 isofluorane in oxygen prior to imaging on an IVIS Lumina II imager (Xenogen, Alameda, CA), as previously
109 described [16]. Quantification of bioluminescence and data analysis was performed using Aura Imaging
110 Software version 4.0.7 (Spectral Instruments Imaging, Tucson, AZ).

111 **T-cell phenotyping**

112 Mouse peripheral blood was obtained, processed and analyzed as previously described [15, 17]. Whole
113 blood was incubated with a major histocompatibility complex I (MHC I) tetramer containing the *T. cruzi*
114 transialidase TSKB20 peptide (ANYKFTLV/Kb) labeled with BV421 (Tetramer Core Facility at Emory
115 University, Atlanta, GA) and the following labeled antibodies: anti-CD8 FITC, anti-CD4 APC EF780, anti-
116 CD127 PE (BD Bioscience, San Jose, CA). At least 500,000 cells were acquired using a CyAn ADP flow
117 cytometer (Beckman Coulter, Hialeah, Florida) and analyzed with FlowJo software v10.6.1 (Treestar, Inc.,
118 Ashland, OR).

119 **Quantitative Polymerase Chain Reaction**

120 For determination of tissue parasite load in mice, samples of skeletal muscle, heart and intestine were
121 collected at necropsy and processed for quantification of *T. cruzi* DNA by real-time polymerase chain
122 reaction (qPCR) as previously described [15, 17, 18]. The limit of detection was set at the lowest
123 standard of 0.0017 parasite equivalents per 50 ng of DNA. For detection of *T. cruzi* infection in dogs,
124 DNA was extracted from 250uL of the buffy coat fraction of EDTA-treated blood using the E.Z.N.A Tissue
125 DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol except 50 µL of
126 elution buffer was used. *T. cruzi*-negative controls (phosphate buffered saline) were included in the DNA
127 extractions. Samples were tested using qPCR for the presence of *T. cruzi* satellite DNA using the Cruzi
128 1/2 primer set and Cruzi 3 probe in a real-time assay, which amplifies a 166-bp segment of a repetitive

129 nuclear DNA [19, 20] as previously described [21] A sample was considered *T. cruzi*-positive if the Ct
130 value was <35 [22].

131 **Multiplex serology**

132 Luminex-based multiplex serological assays were performed as previously described [23, 24]. For a
133 number of smaller proteins, fusions of up to 2 individual genes are employed in order to expand the
134 array of antibodies being detected. (TritrypDb.org identifiers: Antigen **1** (Tc1) = fusion of
135 TcBrA4_0116860 and TcYC6_0028190; **2** (Tc2) = fusion of TcBrA4_0088420 and TcBrA4_0101960; **3** (Tc3)
136 = fusion of TcBrA4_0104680 and TcBrA4_0101980; **4** (Tc4) = fusion of TcBrA4_0028480 and
137 TcBrA4_0088260; **5** (Tc5) = fusion of TcYC6_0100010 and TcBrA4_0074300; **6** (Tc6) = fusion of
138 TcYC6_0043560 and TcYC6_0122760 ; **7** (Tc7) = fusion of TcYC6_0083710 and TcBrA4_0130080; **8** (Tc8)
139 = TcYC6_0037170; **9** (Tc11) = TcYC6_0124160; **10** (Tc17) = fusion of TcBrA4_0028230 and
140 TcBrA4_0029760; **11** (Tc19) = TcBrA4_0122270 and TcBrA4_0131050; **12** (tc2-tol2) = TcBrA4_0101960;
141 **13** (3tolt) = portions of TcBrA4_0101970, TcYC6_0077100 and TcYC6_0078140; **14** (beta-tubulin) =
142 TcYC6_0010960; **15** (G10) = TcCLB.504199.20; **16** (Kn107) = TcCLB.508355.250; **17** (LE2) =
143 TcCLB.507447.19; Parvo = Recombinant Canine Parvovirus VP2 (MyBioSource.com).

144 **Cardiac Troponin**

145 Cardiac troponin (cTnI) analysis was performed using the ADVIA Centaur CP® immunoassay (Ultra-TnI,
146 Siemens Medical Solutions USA, Inc., Malvern, PA) validated in dogs and with a reported range of 0.006
147 to 50.0 ng/mL [25].

148 **Statistical analysis**

149 The non-parametric Mann-Whitney U and unpaired t tests of the GraphPad Prism version 9.4 software
150 were used for the analysis of the mouse experimental data. Values are expressed as means ± standard
151 error of mean.

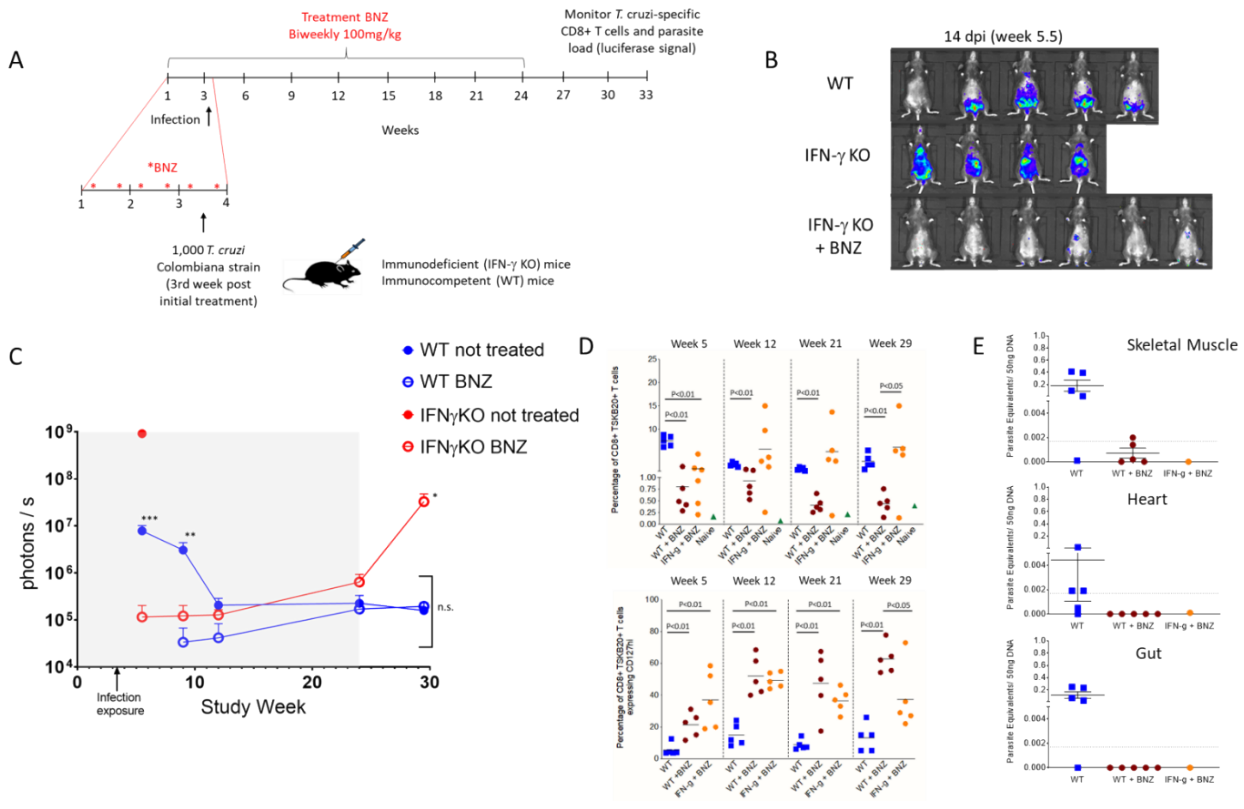
152 **Ethics Statement**

153 The studies in mice were carried out in strict accordance with the Public Health Service Policy on
154 Humane Care and Use of Laboratory Animals and Association for Assessment and Accreditation of
155 Laboratory Animal Care accreditation guidelines. The protocol was approved by the University of
156 Georgia Institutional Animal Care and Use Committee (AUP #A2021 04-011-Y1-A0). Informed consent
157 was obtained from dog owners prior to their participation, and this study was approved by the Texas
158 A&M University Institutional Committee on Animal Use and Care and the Clinical Research Review
159 Committee (IACUC 2018-0460 CA).

160 **Results**

161 We had previously shown that weekly [15] or twice weekly (Bustamante, et al, in preparation)
162 administration of high dose (2.5 – 5 X the normal daily dose) BNZ could cure established infections with
163 *T. cruzi* in mice. To determine if BNZ might also prevent the initial establishment of *T. cruzi* in naïve
164 mice, we conducted a pilot study (Figure 1A) using twice weekly delivery of low dose BNZ (100 mg/kg
165 oral, the standard daily dosage for continuous treatment in mice [26]. At 3.5 weeks (between the 5th
166 and 6th BNZ dose) all mice were injected i.p. with 1000 typomastigotes of the luciferase transgenic
167 Colombiana strain of *T. cruzi*. Groups of both wild-type (C57BL/6J) and IFNγ deficient mice were
168 included in the study; the IFNγ deficient (KO) mice are highly susceptible to *T. cruzi* infection [15, 27] and
169 were expected to reveal even very low levels of persistent infection. Untreated mice in both groups
170 exhibited evidence of active infection at 14 days post-exposure and beyond and as expected, all mice in
171 the IFNγ deficient group not receiving prophylaxis experienced uncontrolled infections and had to be

172 euthanized by ~21 days post-exposure. In contrast, some BNZ-prophylaxed IFN-g KO mice showed low
173 level luciferase signal at day 14 post-exposure but not at 12 weeks, and none of the mice in the BNZ-
174 treated WT group had a detectable luciferase signal above background throughout the study (Fig 1B, C
175 and Fig. S1).



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177 Figure 1. **(A)** Schematic representation of prophylactic treatment, infection, and monitoring of mice. WT and IFNγ
178 deficient mice were treated biweekly with 100mg/kg of BNZ over 24 weeks. After the second week of treatment,
179 mice were infected (5-6 animals/group) intraperitoneally with 10^3 trypomastigotes of the Luciferase-expressing
180 Colombiana strain of *T. cruzi*. Control groups of mice were infected and left untreated. **(B)** Representative images
181 showing bioluminescent signal in mice (ventral view) at 14 days post-infection exposure. **(C)** Parasite
182 bioluminescence quantification following D-luciferin injection was measured at the indicated times in the study
183 period. Each data point represents the mean of bioluminescence from 3-6 mice expressed on a logarithmic scale as
184 mean photons/second ±/- standard error of the mean. Grayed box marks the prophylaxis period. * $p<0.03$ relative
185 to both WT groups; ** $p<0.008$ relative to both BNZ-treated groups; *** $p<0.005$ relative to BNZ-treated IFNγ KO

186 group; n.s. bracketed data points are not statistically different. **(D)** Percentage of $CD8^+$ blood lymphocytes specific
187 for the *T. cruzi* TSKb20 epitope (top) and expressing the T cell central memory marker CD127 (bottom) during the
188 study course **(E)** *T. cruzi* DNA determined by quantitative real time polymerase chain reaction in the skeletal
189 muscle, heart, and intestine of untreated and BNZ-treated mice at week 33 of the study (~30 weeks post-
190 infection).

191 Infection exposure was also confirmed in mice by monitoring the generation of *T. cruzi* -induced $CD8^+$ T
192 cells specific for the immunodominant TSKb20 epitope [26]. With the exception of one prophylaxed
193 IFNγ KO mouse, all mice exhibited *T. cruzi* -specific $CD8^+$ T cell responses with comparable numbers in
194 the untreated WT and prophylaxed IFNγ KO mice and lower levels in the prophylaxed WT mice. We
195 have previously shown that the expression of the T cell central memory marker CD127 on these
196 parasite-specific T cells is a useful surrogate for parasite load [15, 26] and the 2 groups under
197 prophylaxis had a substantially higher fraction of $CD127^+$ cells in the TSKb20-specific population relative
198 to mice not receiving prophylaxis, consistent with the expected low/absent parasite burden through the
199 21st week of prophylaxis (Fig 1D).

200 Twice weekly BNZ was continued for a total of 24 weeks, simulating a seasonal period of vector activity
201 typical of south Texas [13]. In WT mice with or without prophylaxis, infection was not readily detectable
202 by whole animal imaging through the prophylaxis period. However, in the IFNγ KO mice, although the
203 infection was largely controlled by the prophylaxis treatment, the systemic infection was barely
204 detectable during the prophylactic treatment period (Fig S1) and within 4 weeks of termination of BNZ
205 prophylaxis, 4 of 5 mice in the previously treated IFNγ KO mice group were systemically parasite-positive
206 and had to be euthanized (Figure 1C and Sup Figure 1). The remaining animals in all groups were
207 euthanized at week 33 of the study and skeletal muscle and cardiac tissues were examined for the
208 presence of *T. cruzi* using qPCR. As shown in Fig 1E, all of the untreated WT mice were PCR positive for
209 *T. cruzi* DNA in one or more of the samples from skeletal muscle, heart, or gut. In contrast, at least 3 of

210 the 5 WT mice receiving BZN prophylaxis and the surviving IFNγ KO mouse were negative for *T. cruzi*
211 DNA (the remaining 2 WT-treated mice were at or below the level of detection in this assay in skeletal
212 muscle only). Collectively, these results suggest that the prophylaxis regimen employed here in WT mice
213 prevented or truncated an infection in most cases. However, the protective effect of prophylaxis
214 generally required a coincident intact immune response, as most IFNγ deficient mice became and
215 remained infected despite prophylaxis.

216 Building upon these encouraging results in mice, we next asked if BNZ prophylaxis could reduce
217 infection in kennel and hunting dog populations in the south-central U.S. where infection pressure was
218 high, despite other vector control efforts [11]. We conducted an early spring (March to April) screening
219 to identified uninfected dogs in this setting with the intent of capturing infection status prior to the
220 window of high vector activity [13], using a combination of negative serological tests and negative blood
221 PCR. The rationale for using this combination of tests is that although blood PCR can provide solid
222 evidence of active infection, a negative blood PCR assay is not a dependable indicator of absence of
223 infection. Additionally, serologic tests may miss very recent infections. A total of 126 dogs of previously
224 undetermined infection status were screened using a Luminex-based assay previously employed for
225 detection and monitoring *T. cruzi* infection in humans [28-30] and other species [24, 31], a commercial
226 Stat-Pak test (ChemBio, NY) that is validated for humans but commonly used for research purposes in
227 dogs [32, 33], and by blood PCR. Figure 2A displays dogs considered to be infection-negative based
228 upon the Luminex assay and confirmed in most cases by Stat-Pak and indirect fluorescent antibody (IFA)
229 (Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX; [32]) assays (Supplemental
230 Table 1; n=57; 45.2% of total screened). Nearly all dogs had detectable antibodies to the parvovirus
231 vaccine antigen, confirming the quality of the serum sample, and in all cases the negative *T. cruzi*
232 serology was supported by a negative blood PCR assay.

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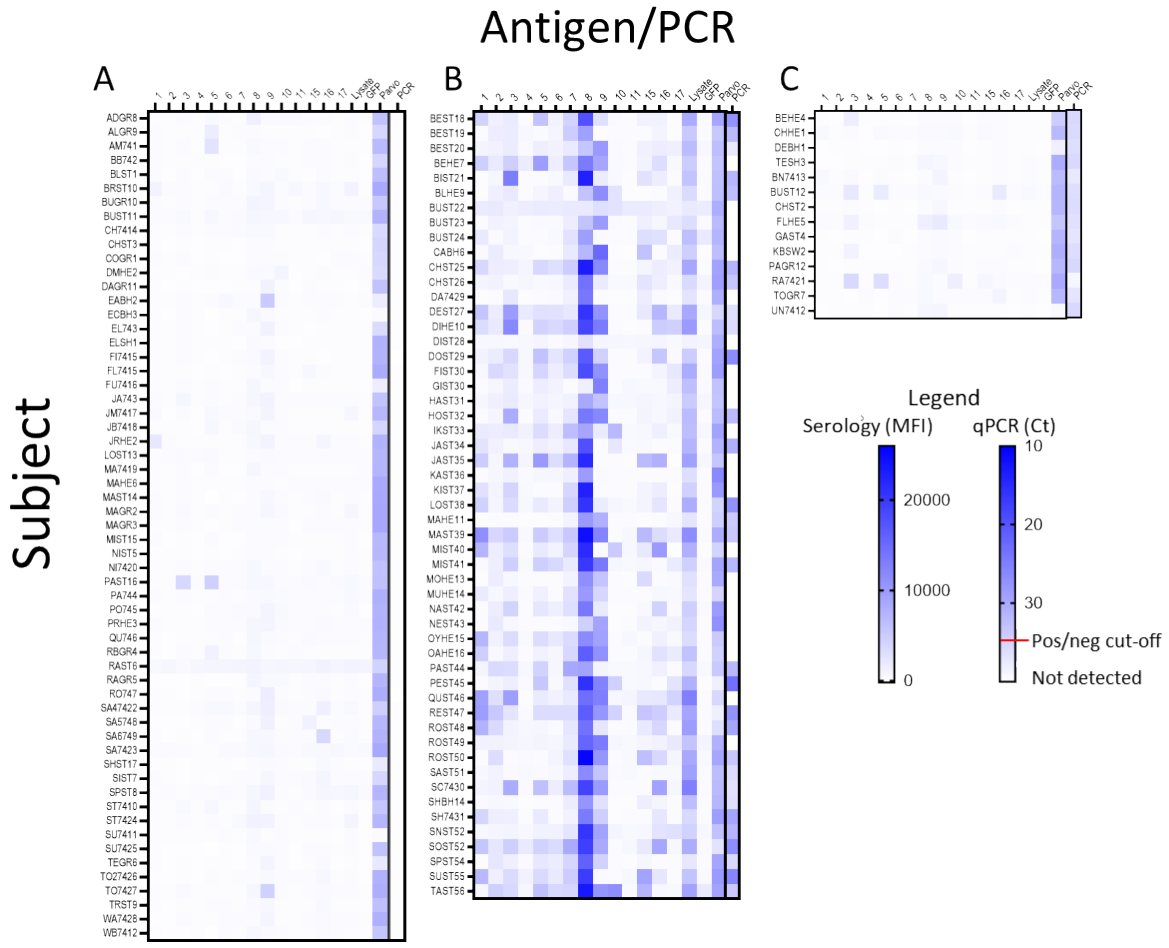

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235 Figure 2: Initial screening results with multiplex serology and PCR to identify *T. cruzi*-negative dogs to enroll in the

236 prophylaxis study. Heatmaps of antibody levels (mean fluorescence intensity; MFI) and qPCR measurement of *T.*

237 *cruzi* DNA in blood were used to classify dogs as uninfected **(A),** *T. cruzi*-infected **(B)**, or seronegative but with a

238 blood PCR result that did not reach the cut-off for being considered positive **(C)**. Recombinant *T. cruzi* proteins are

239 defined in the Materials and Methods; lysate = a sonicate of *T. cruzi* trypomastigotes and amastigotes;

240 GFP=recombinant green fluorescent protein (negative antigen control); Parvo = Parvovirus vaccine antigen

241 (positive serological control).

242 Figure 2B shows the antibody and/or PCR+ dogs that were excluded from the prophylaxis trial by virtue

243 of having prior exposure and likely active infection (n=53; 42.1% of those screened). Roughly half (29/53;

244 54.7%) of the dogs with detectable (generally robust) serological responses also had *T. cruzi* DNA in their
245 blood as determined by qPCR. It is notable that, as in other species [24, 28-31], the pattern of antigen
246 specificity of antibody responses in these subjects varied extensively, although the antigen in lane 8, a
247 polyubiquitin protein, appeared to be the most reliable. A fraction of the screened dogs (n = 14; 11.1%)
248 were seronegative by Luminex and other assays but had a weak signal in the blood PCR assay that was
249 measurable, but above our standard cutoff for determining infection (Ct < 35.0; Fig 2C and Table S1).
250 These dogs were initially enrolled in the prophylaxis study as uninfected but 4 of these likely were
251 already infected and these were excluded from prophylaxis study analysis (detailed further below).

252 The 67 animals considered infection-negative and available for enrollment were randomly assigned to
253 either a control (no therapy) or a prophylactic group, which received twice weekly doses of BNZ at a
254 level of not less than 10 mg/kg. This dosage is approximately the normal **daily** dose previously used
255 (mostly ineffectively) to attempt drug-cure in dogs [34-36]. Dogs in these groups were then re-sampled
256 10-12 weeks (considered the midpoint of the transmission season) and ~24 weeks (end of season).

257 Apparently new infections occurred in both the control (n=6; 18.2%) and prophylaxis (n=9; 26.5%)
258 groups, yielding a combined seasonal infection incidence of 22.4%. In all but one case, these infections
259 occurred early in the season, prior to the 12 week screening timepoint. One newly-infected dog in each
260 group was lost to follow up before the 24 week sampling point (one was sold and one died due to *T.*
261 *cruzi* with severe, acute, lymphohistiocytic, necrotizing pancarditis with intralesional amastigotes). Additionally,
262 one infected dog in each group was removed from the study after the mid-point sampling due to high
263 serum cardiac troponin I (cTnI) levels (indicative of active heart damage) and started on a treatment
264 protocol consisting of higher dose BNZ (to be reported on elsewhere). Thus, BNZ prophylaxis as
265 employed in this study had no impact on the number of new infections in this high intensity
266 transmission setting.

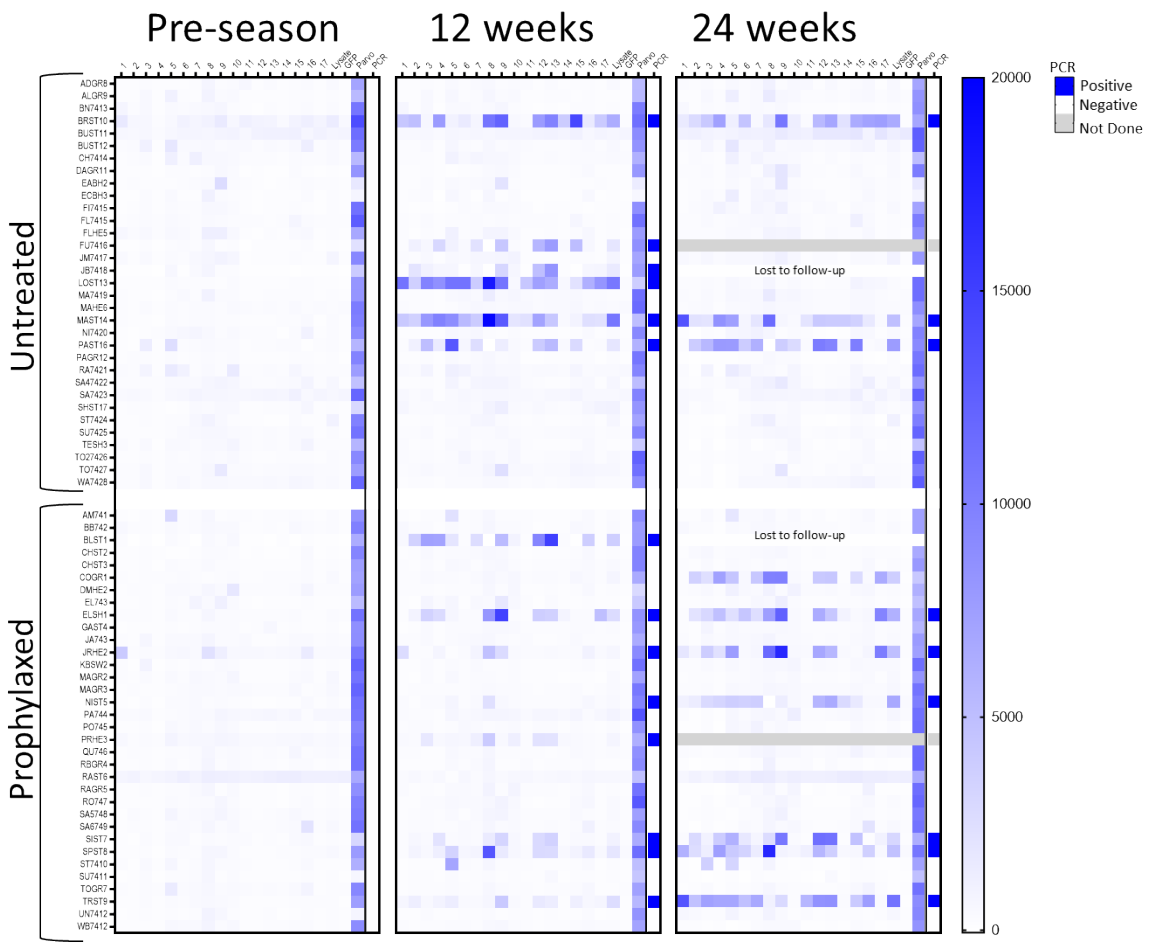

Figure 3: Multiplex serological and blood PCR changes over the transmission season in dogs receiving or not BNZ prophylaxis. Serological and PCR negative dogs (see Figure 2) were randomly assigned to the untreated or BNZ treatment groups and then rescreened at ~12 and ~24 weeks after the beginning of prophylaxis. One dog in each group (gray fill) was moved to BNZ treatment protocol after the 12 week sampling point due to high serum cardiac troponin I (cTnI) levels. One dog in each group was lost to follow-up before the 24 week sampling time due to death or sold by owner. Antigens used in the multiplex assay are as described in Figure 2 and the Materials and Methods. Blood PCR was considered positive if below the cut-off Ct value of 35.

The serial sampling during a transmission season and the stable pattern of the antibody profiles in infected subjects provided the opportunity for several additional novel observations. First, 4 of the 16 seronegative dogs with blood PCR signals exceeding the positive cutoff point (Figure 2C) developed a

seropositive profile during follow-up (Figure 4) and 3 of the 4 developed strong blood PCR signals. While these could represent new infections acquired during the follow-up period, they may also indicate dogs that were already infected at the time of the March/April survey but were so early in the infection course that antibody responses had not developed and parasites in the blood were low. The remaining 12 dogs in this subset were equally dispersed in the untreated and prophylaxis groups and none developed evidence of infection during the 24-week follow-up (Figure 3 and Table S1). Looking retrospectively, one dog in each group in the prophylaxis trial was PCR negative (JRHE2, prophylaxis group and PAST16, control group) but was nonetheless also likely already infected at the pre-season survey point, as both had low reactivity to several*T. cruzi* antigens in the multiplex assay at this point but with a pattern that matched the more robust antigen recognition pattern in the Luminex assay that ultimately developed by the 12 and 24 week sampling points (Figure 3).

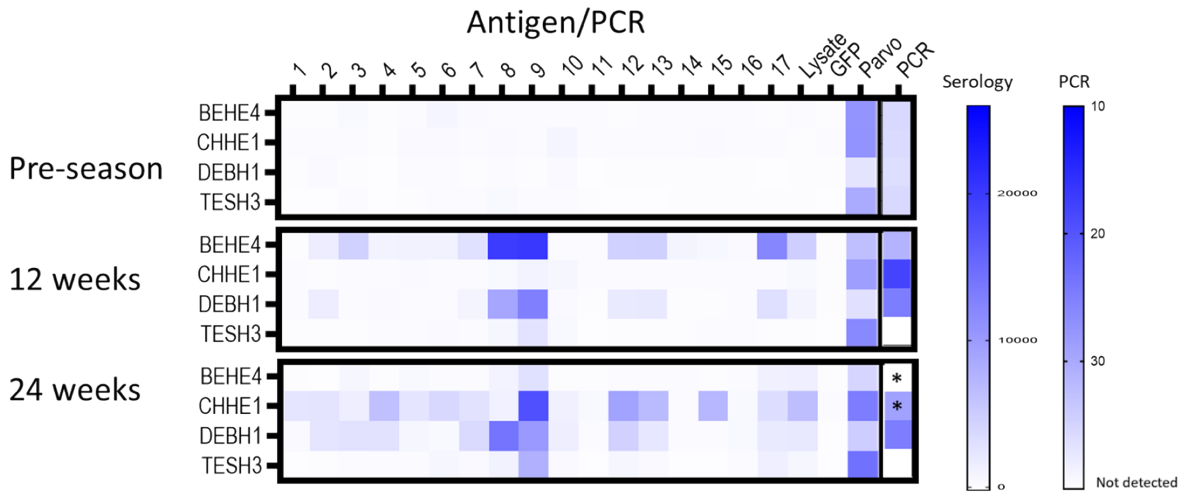

Figure 4. Multiplex serological and blood PCR changes over the transmission season in dogs that were seronegative but PCR positive in the pre-transmission season screening (see Figure 2). Antigens used in the multiplex assay are as described in Figure 2 and the Materials and Methods. * moved to a high dose BNZ treatment protocol after 12 week sample point.

294 Lastly, one dog in the control group (LOST13, Figure 3) had developed a substantial antibody profile by
295 the 12 week sampling point (at which he was also PCR positive), but had essentially totally lost this
296 response by the 24 week screening point. This dog was also strongly positive by a StatPak antibody test
297 and had detectable serum cTnI (0.021 ng/mL) at 12 weeks but was seronegative and had cTnI below
298 lower detection limit of 0.006 ng/mL at the 24 week sample time point. The continued detection of
299 parvovirus-specific antibodies over this same time course ruled out concerns on sample quality or
300 immune competency. A similar pattern of cure was evident in dog BEHE4 (Figure 4) after he was
301 switched to a BNZ treatment regimen (increasing the dosage to 30 mg/kg twice a week) due to high cTnI
302 levels in the 12 week sample. This dog became PCR negative and seronegative by the 24 week sample
303 point. Collectively, these data support a potential early infection spontaneous cure one dog and BNZ-
304 induced cure in another, both resulting in a rapid decline in serological evidence of the previous
305 infection.

306 **Discussion**

307 Our previous studies have extensively documented the high risk of *T. cruzi* infection in dogs in south
308 central Texas, including among kenneled working dog populations [11, 21, 32]. New infection rates of
309 up to 30%/year put these valuable animals at high risk for morbidity and early death. It is noteworthy
310 that this high incidence of infection is occurring despite vector awareness education for dog owners and
311 the implementation of a variety of vector control measures. Likely, the abundance of insect vectors and
312 wildlife reservoirs with active *T. cruzi* infections, the outdoor group housing conditions that expose dogs
313 to this infection risk, and the propensity of dogs to ingest these vectors and their feces, all combine to
314 promote this high transmission situation. The lack of interventions such as vaccines that could reduce
315 the incidence of new infections and the high failure rates of potential therapies leave few options for
316 reducing the impact of *T. cruzi* in these settings.

317 In this study, we explored the potential of BNZ, a drug that has been in use for >50 years for the
318 treatment of *T. cruzi* infection, in a prophylactic modality to attempt to reduce the rate of new
319 infections in dogs. To our knowledge, prophylactic use of trypanocidal drugs has not been previously
320 explored for prevention of new infections with *T. cruzi* [37], although prophylaxis has occasionally been
321 employed to prevent potential exacerbation of infection due to immunosuppression following tissue
322 transplantation in humans [38]. The premise behind these studies was that dosing BNZ at a standard
323 daily dose level, but administering it twice per week instead of daily, could prevent establishment of
324 new infections, or might rapidly terminate those infections before they could become firmly established.
325 The twice-weekly dosing pattern is supported by the ability of BNZ to cure established *T. cruzi* infections
326 in mice using this schedule, albeit with 2.5-5-fold the dose level used here for prophylaxis. Selection of
327 this lower prophylactic dose, bi-weekly schedule was also driven by the practicality in terms of
328 time/effort, cost, and cumulative drug toxicity over a potential 6-month transmission season.

329 Trials of BNZ-based prophylaxis in laboratory mice yielded promising results, demonstrating a
330 substantial reduction in parasite burden and the ultimate resolution of infection in the majority of
331 immunocompetent mice. Notably, this protocol had much less impact in mice compromised in the
332 ability to produce IFNγ, an essential component of the anti-*T. cruzi* immune response. This latter result
333 supports the hypothesis that a competent immune response likely works in concert with trypanocidal
334 drugs to ultimately clear *T. cruzi* infection when treatment is successful.

335 However, in dogs in a high transmission setting, prophylaxis as applied here failed to prevent new
336 infections during a 6-month transmission season. A number of factors could have contributed to this
337 differential result in mice and dogs, primary among them, the likely differential parasite exposure. Mice
338 received a single sub-lethal intraperitoneal injection of 1000 trypomastigotes, while dogs were
339 potentially exposed repeatedly to infection, possibly at much higher doses, and likely via an oral route.
340 The fact that one dog newly infected during the died during the acute infection and others exhibited

high serum cTnI levels, prompting their transfer to a higher treatment dose regimen, supports the higher (and also variable) exposure conditions for dogs in this study. Effective prophylaxis under these scenarios might require a more aggressive dosing regimen, either more frequent or with higher dosage or both.

Although not designed to explicitly address this point, the infection pattern observed in this study supports a seasonality in infection potential. We estimated the transmission season based upon cumulative vector sighting observations collected by our Kissing Bug Community Science initiative [13], which suggested vector activity beginning in May and peaking in July. However, at least 4 dogs were borderline PCR positive but seronegative in early May, and all developed serological responses, 3 of the 4 within 10-12 weeks of the initial sampling. Additionally, two dogs that were PCR-negative, but had a suggestion of a serological response at initial screening, were PCR positive 10-12 weeks later. Collectively, these data indicate that new infections may be occurring before our early May prophylactic dosing. In combination with the fact that most new infections occurred in the first 12 weeks dosing period, it seems likely that beginning prophylaxis earlier in the year could have had a greater impact in the rate of new infections. Including these four “pre-prophylaxis” infected dogs as among those infected in 2021 year increases the yearly incidence in this setting to 26.8% (19/71).

To our knowledge, no previous study has sampled a susceptible, at risk, animal population at the frequency and with both PCR and multiple serology as employed in this study. In addition to the high infection pressure, this setting is also optimal for collection of novel and valuable data because new dogs are introduced each season, through either breeding or new acquisitions. Among the interesting observations from this first screening year is that 14 of the 15 (or 18 of 19 if the 4 pre-May infections are included) new infections occurred before the calculated mid-point of the estimated transmission season. The reason for this early season bias is not known, but could be due to opportunity based on location, or to behavior (e.g. aggressive bug eaters are more likely to be exposed and become infected)

365 or a combination of the two. Given the high early season incidence, it is clear that the opportunities for
366 infection are high even when bug activity appears low. Identifying the specific links between bug
367 numbers, locations and infection status with the seasonality of new infections in dogs should be
368 addressed in future seasonal studies. Active entomological surveillance at sentinel locations within the
369 geographic region of study would be useful to establish a more precise start of the seasonal insect
370 activity period, so that prophylaxis in future years could be initiated prior to insect activity.

371 The frequency of sampling applied herein also allowed for the detection of one apparent case of
372 spontaneous cure of an acute infection. Although such spontaneous cures have been anecdotally
373 reported in chronically infected hosts, both animal [39] and human [40], to our knowledge cure during
374 the acute infection has not been documented. The frequency of such cures is worth exploring further
375 and as well, the protection from re-infection that might be afforded by such cases. The very rapid
376 waning of the antibody response in this case, as well as following an apparent BNZ treatment dose-
377 induced cure, was also surprising and would have gone undetected if screening was conducted only
378 once or twice per year, for example.

379 The use of both of a multiplex serological test and blood PCR to track new infections was critical to the
380 technical success of this project and for revealing some of the less expected observations. The frequent
381 failure of PCR to detect many chronic infections with *T. cruzi* is well recognized [31]. The multiple
382 antigen array used in this study, incorporating >25 parasite proteins, a crude lysate, and both positive
383 and negative control proteins, provides a confidence in detection of infection that is lacking in single
384 antigen or whole parasite assays. However, all serological assays fail infection detection very early,
385 before anti-parasite antibodies have been formed. Thus, the combined use of PCR and multiplex
386 serology provided insights that would have been missed using either approach alone.

387 Also, not addressed in this study is whether prophylaxis, while not reducing the incidence of infection,
388 might be beneficial by ultimately reducing the severity of disease. Anecdotally we observed that several
389 dogs infected while under prophylaxis nonetheless experienced high serum levels of cTnI, indicating that
390 prophylaxis in these cases was not preventing acute phase disease. A longer-term follow-up of disease
391 development with and without early prophylaxis might be revealing. However, ideally, when infection is
392 detected in these working dogs, they should be enrolled in an effective treatment regimen (as done for
393 dogs BEHE4 and CHHE1) that enhances the chances of resolving the infection and thus preventing
394 disease progression.

395 Although the seasonal monitoring for new infections in a population at high risk of infection provided
396 new insights into *T. cruzi* transmission and development of immune responses, the prophylaxis
397 approach applied here did not prevent infection in this setting. Future studies might apply prophylaxis
398 earlier in the transmission season or at a more aggressive dosing level. Also, it would be of interest to
399 determine if prophylaxis, while failing to reduce infection incidence, might be beneficial with respect to
400 reducing infection impact either in the short-term, or long-term.

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557

558 **Supporting Information Captions**

559 **Supplemental Figure 1**. Images of *T. cruzi* bioluminescent signal in mice (ventral view) at various times
560 post study initiation (see Figure 1A) Note: only 3 of the 5 mice in WT groups were imaged at 24 weeks.

561 **Supplemental Table 1**. Pre-study dog survey data

562