Title: Serial 'deep-sampling' PCR of fragmented DNA reveals the wide range of *Trypanosoma cruzi* burden among chronically infected hosts and allows accurate monitoring of parasite load following treatment

Short title: 'Deep-sampling' PCR of fragmented DNA for sensitive *T. cruzi* detection

Brooke E. White[1], Carolyn L. Hodo[3], Sarah A. Hamer[4], Ashley B. Saunders[5], Susana A. Laucella[6], Daniel B. Hall[7], Rick L. Tarleton[1,2,8]

[1]Center for Tropical and Emerging Global Disease and [2]Department of Cellular Biology, University of Georgia, Athens, Georgia, USA

[3]Michale E. Keeling Center for Comparative Medicine and Research, The University of Texas MD Anderson Cancer Center, Bastrop, Texas, USA

[4]Department of Veterinary Integrative Biosciences, School of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA

[5]Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, USA

[6]Research Department, Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", Buenos Aires, Argentina. Chagas Disease Unit, Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina

[7]Department of Statistics, University of Georgia, Athens, Georgia, USA

[8]Corresponding author: tarleton@uga.edu

Abstract

Infection with the protozoan parasite *Trypanosoma cruzi* is generally well-controlled by host immune responses, but appears to be rarely eliminated. The resulting persistent, low-level infection results in cumulative tissue damage with the greatest impact generally in the heart in the form of chagasic cardiomyopathy. The relative success in immune control of *T. cruzi* infection usually averts acute phase death but has the negative consequence that the low-level presence of *T. cruzi* in hosts is challenging to detect unequivocally. Thus, it is difficult to identify those who are actively infected and, as well, problematic to gauge the impact of treatment, particularly in the evaluation of the relative efficacy of new drugs. In this study we employ DNA fragmentation and high numbers of replicate PCR reaction ('deep-sampling') and to extend the quantitative range of detecting *T. cruzi* in blood by at least 3 orders of magnitude relative to current protocols. When combined with sampling blood at multiple time points, deep sampling of fragmented DNA allowed for detection of *T. cruzi* in all infected hosts in multiple host species. In addition, we provide evidence for a number of characteristics not previously rigorously quantified in the population of hosts with naturally acquired *T. cruzi* infection, including, a > 6-log variation between chronically infected individuals in the stable parasite levels, a continuing decline in parasite load during the second and third years of infection in some hosts, and the potential for parasite load to change dramatically when health conditions change. Although requiring strict adherence to contamination-prevention protocols and significant resources, deep-sampling PCR provides an important new tool for assessing new therapies and for addressing long-standing questions in *T. cruzi* infection and Chagas disease.

Author Summary

Infection by the protozoan *Trypanosoma cruzi* normally results in a life-long, but low-level parasitization of muscle tissues, often leading to chagasic heart disease. A major challenge in the Chagas disease field has been the difficulty in detecting and quantifying parasite load in infected hosts. In this study we show that collection of serial blood samples and performance of sometimes high numbers of replicate PCR reactions on fragmented blood DNA, allows detection and quantification of relative parasite load in non-human primates, dogs, and humans with naturally acquired *T. cruzi* infection. This 'deep-sampling' approach reveals a mostly stable, 100,000-fold or greater difference in parasite load among chronically infected hosts and can detect alterations in parasite levels due to changes in health status or following therapeutic treatment in individual hosts, thus providing a powerful tool for assessing treatment outcomes in *T. cruzi* infection, including for evaluation of new therapeutics. Additionally, the ability to accurately and sensitively monitor parasite load in hosts provides the means to address highly contentious issues in the Chagas field, including the relative role of parasites and hosts in establishing the persistent parasite burden and the relationship between parasite burden and the presence and severity of clinical disease.

Introduction

Chagas disease, the result of infection with the protozoan *Trypanosoma cruzi,* is endemic to the Americas, where it is among the highest impact infectious diseases and is also a major source of infection-related heart disease globally. Chagas disease is a result of the long-term persistence of *T. cruzi* primarily in muscle tissues, despite highly effective immune responses that generally control but fail to completely clear the infection in the majority of individuals. Although *T. cruzi* continuously alternates between replicating forms inside host cells and non-replicating forms in extracellular spaces, including the bloodstream where it can be acquired by blood-feeding triatomine insects, detection of parasites or parasite products in the blood is generally undependable, using even the most sensitive methods. Consequently, diagnosis of infection generally rests mainly on serological tests, which are often not fully reliable.

Positive serological tests reflect prior exposure but not necessarily active infection. Thus, determining the effectiveness of current treatments to clear the infection or whether some subjects have spontaneously resolved the infection (apparently rare, but anecdotally reported) and thus should not be treated, remains out of reach. The inability to routinely and sensitively detect active infection coupled with the undependable curative capabilities of current therapeutics and their high levels of side effects, accounts for the estimates that less than 1% of *T. cruzi*-infected individuals receive anti-parasitic treatment [1]. Furthermore, the lack of sensitive methods to definitively establish cure hinders the identification and validation of improved therapies.

Amplification techniques such as PCR can specifically enrich very low quantities of pathogen DNA and have been extensively used to enhance the detection of *T. cruzi* DNA in the blood of infected hosts. Multiple, high copy number (>10^5 copies per organism) targets for amplification have been identified and rigorous, highly specific amplification protocols have been developed and evaluated [2]. Nevertheless, it is generally agreed that these protocols, as currently employed, frequently fail to detect *T. cruzi* in infected hosts and thus are not reliable tests of the absence of infection. Multiple studies have attempted to address the sensitivity and specificity of the *T. cruzi* PCR methodology, for example, varying the target (there are 2 primary ones used, a kDNA and a genomic satellite sequence), primer and probe sequences, DNA storage and purification techniques (automated or not), and volume of blood drawn for DNA isolation (1-10 ml) [3, 4]. In general, none of these variations significantly alter test outcomes. What does alter the ability of PCR to detect *T. cruzi* infection is the number of independent blood collections done and the number of PCR determinations conducted for each sample [5].

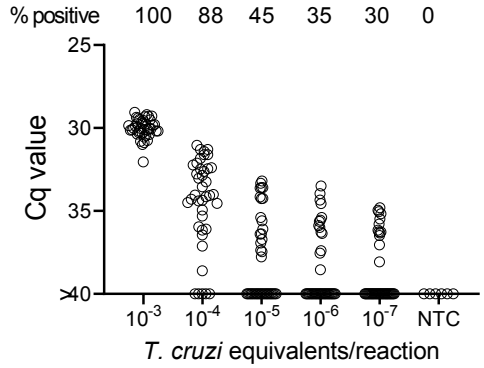
These results highlight that detection of *T. cruzi* DNA in infected hosts is frequently a problem of sampling. Specifically, when pathogen numbers are low, collection of a high number of test samples that are extensively sub-sampled may be required in order to have an opportunity to detect a pathogen. For *T. cruzi*, this sampling problem is strongly supported by the remarkable study done in the 1970's by Cerisola in Argentina [6] in which 30 untreated subjects submitted to approximately monthly xenodiagnosis with 80 triatomines each month for >2 years. This study showed that some individuals had multiple bugs positive at every sample point (thus, presumably a high parasite load) while others were more variable (strongly positive some months but not others), and a few were only very occasionally positive (in the most extreme case, as few as 2 positive bugs out of >1000 fed over 24 months).

100 In the current study, we have used humans, non-human primates (NHP), and dogs, all with naturally
101 acquired *T. cruzi* infection, to demonstrate that serial sampling of blood and exhaustive PCR of optimally
102 prepared DNA from that blood, can confirm even the most difficult to detect infections with *T. cruzi*. The
103 ability to obtain relative quantification of parasite load in these naturally infected hosts reveals the wide
104 range of parasite load among infected subjects in a population (> 6 $\log_{10}$) and the conditions under
105 which parasite control can change, both slowly in the early years of infection and dramatically when
106 overall health changes. Deep-sampling PCR, while laborious, offers the first true test of cure for Chagas
107 disease and should also assist in the determination of associations between parasite load, immune
108 response parameters, and the risk for disease development in chronically infected hosts.

109 **Results**

110 Performing large numbers of replicate PCR reactions ('deep-sampling') extends the range to detection of
111 *T. cruzi* infection in naturally infected hosts.

112 In order to determine the potential of deep-sampling for more sensitive detection of *T. cruzi* in blood
113 samples, we spiked macaque blood with DNA equivalent to known numbers of parasites and conducted
114 ~40 replicate PCR reactions per DNA sample. A blood DNA aliquot for PCR amplification equivalent to
115 10^{-3} parasites per reaction was selected as the highest parasite concentration as this is the lowest
116 standard that consistently provides a positive signal in our standard PCR assay. This result was
117 confirmed in this assay when all 40 aliquots expected to contain 10^{-3} parasites were positive for *T. cruzi*
118 DNA amplification (Fig 1). Decreasing the parasite equivalents (PE) in 10-fold increments expectedly
119 reduced the frequency of positive PCR reactions and as well, the maximum Cq value of each positive
120 amplification set, but demonstrated that as few as 10^{-7} PE per reaction could be detected if a sufficient
121 number of replicate reactions were conducted PCR.



122

123 Figure 1. Deep-sampling (replicate PCR) allows detection of *T. cruzi* in a decreasing frequency of
124 replicate reactions to at least 4 orders of magnitude below the normal limit of quantitation (10^{-3}) used
125 for single PCR reactions. % positive is the percentage of replicate reactions that gave a detectable
126 amplification in 40 cycles (Cq value <40). NTC = no *T. cruzi* DNA

127 We next turned to the use of *bona fide* blood samples from *T. cruzi* infected hosts to test the ability of
128 deep-sampling PCR to detect *T. cruzi* DNA in naturally infected hosts with an expected variability of
129 circulating parasites. Non-human primates in indoor/outdoor housing in the southern U.S. readily
130 acquire *T. cruzi* by environmental exposure to the often plentiful infected triatomine insects present in
131 these settings [7] and maintain immunologically controlled but persistent infections similar to that in
132 humans [8, 9].

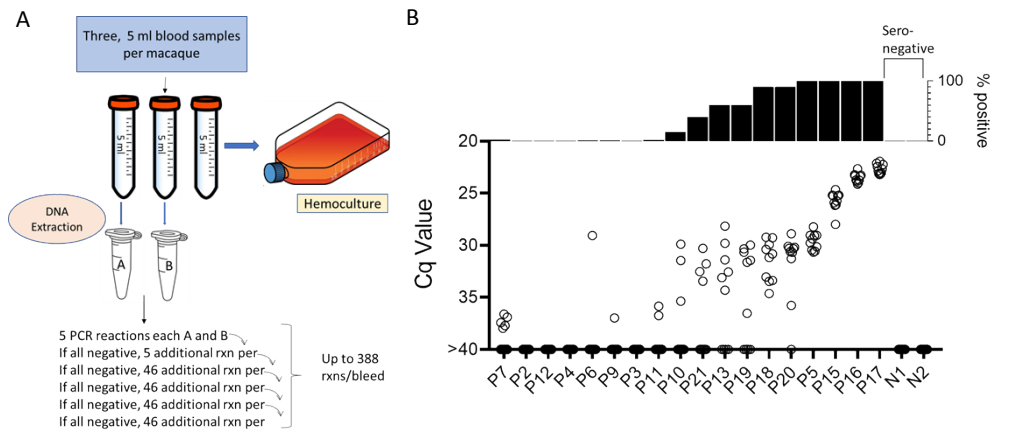


Figure 2. A. Protocol for the collection and PCR analysis of NHP blood samples. B. The combined PCR results of samples A and B at the first of monthly sampling points, plotting Cq values for all replicates (bottom) and percent positive replicates (top).

133

134 In total, 21 *T. cruzi* seropositive, two seronegative, and 5 previously treated and cured [8] rhesus
135 macaques were used in the study. The average age of the seropositive macaques was 13.4 yrs (range 8-
136 24) and the presumed period of infection (based upon first positive serology in annual sampling) was
137 between 2 and 11 years (mean = 5.7) (full data on all the animals is provided in S1 Table).

138 Samples were collected and processed as shown in Fig 2a. In brief, 3 approximately 5ml blood samples
139 were collected from the same needle stick and DNA was isolated from two of the whole blood samples
140 and the 3rd sample was submitted to hemoculture. PCR amplification of replicate 125 ng samples of the
141 blood DNA was conducted for each of samples A and B as noted until a positive reaction was observed in
142 either sample or until the DNA was depleted, up to a total of 194 amplification reactions per sample. In
143 the initial sampling point, 11 of 18 (61%) seropositive macaques had at least 1 positive amplification in
144 10 or fewer PCR reactions and 6 of these 11 were positive on 9 or 10 of the 10 reactions and thus likely
145 to be detected by a standard PCR (one or two replicate) test Figure 2b). On the other extreme, 4 of 18
146 (22%) seropositive macaques were PCR negative despite as many as 388 replicate reactions. Likewise,
147 blood DNA from seronegative controls was negative in a >300 replicate reactions (S1 Table).

148 To determine the constancy of detectable parasite DNA in blood over time, blood samples were
149 collected monthly for up to 1 year and processed as in Figure 2a. Profiles of representative animals over

150 this year of sampling are shown in Figure 3; the full data set for all 28 animals involved in the study is
151 shown in S1 Fig.

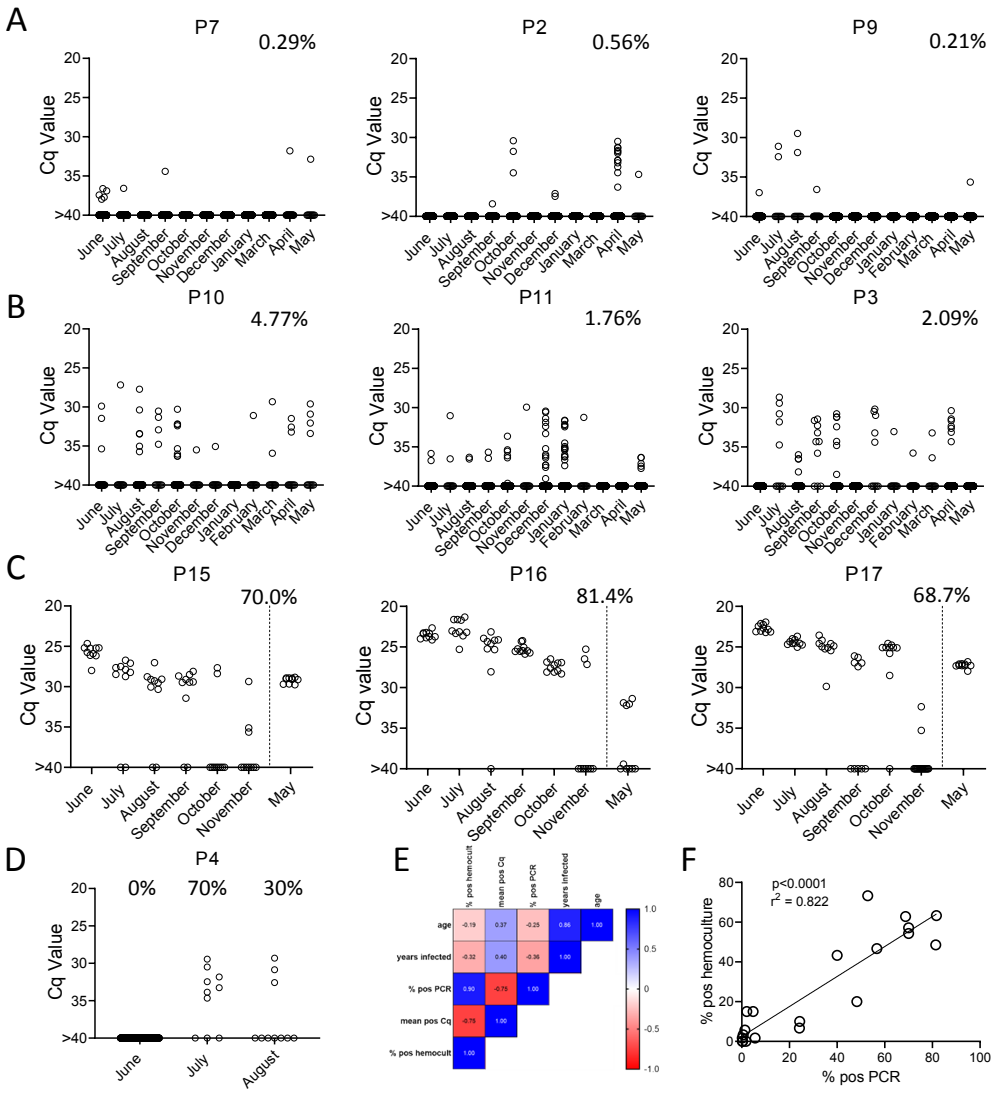


Figure 3. Monthly tally of representative macaques with A) rarely, B) variably or C) frequently positive PCR reactions over the one year of sampling. Animals in groups A) and B) were sampled all 12 months while those in C) were sampled only for the first 6 months then in the 12th month. Percentages indicate the overall percentage of positive PCR reactions over all sampling points. D) Macaque P4 switched from 100% negative to 50% positive PCR reactions coincident with a change in health status. E) and F) Pearson correlation analysis indicated a strong positive correlation between the overall frequencies of positive PCRs and hemocultures and a negative correlation between these frequencies and Cq values, but no significant correlation between age or length of infection with any of the three parasite parameters.

152 All 21 seropositive macaques had one or more positive PCR reaction at one or more of the sample points
153 over the initial 12-month study period. Figures 3a, 3b and 3c show a representative set of animals in
154 which PCR reactions were respectively very rare, variable or frequent. Macaques P7 and P9 were two of
155 the most extreme cases in terms of a low frequency of positive PCR reactions, with respectively 7
156 (0.21%) and 10 (0.29%) positive reactions from a total of >3000 reactions performed on 24 blood
157 samples each. The example macaques shown in 3b had higher frequencies of PCR positive reactions
158 (1.76, 2.09, and 4.77) and with lower Cq values, but also had occasional months with no positive
159 detection. Collectively 8 of the 21 seropositive macaques had 1 or more months in which as many as
160 388 replicate PCR reactions failed to detect an amplifiable product. Pearson correlation analysis
161 revealed a very strong negative correlation between the overall frequency of positive PCR reactions in
162 animals and the average Cq values for those positive reactions (low Cq values represent higher target
163 DNA; Fig 3e). All macaques screened for a minimum of 12 months in the first year of the study or the
164 eight additional bleed points in the second year (see below) were positive by hemoculture in one or
165 more samples with one exception and the percent positive hemoculture correlated strongly with both
166 the frequency of positive PCR reactions and the average Cq values of positive PCRs (Fig 3e and 3f). Thus,
167 assessing the frequency of positive PCR reactions among deep-sampled, serially collected blood-derived
168 DNA blood allows highly sensitive and quantitatively accurate determination of infection status and
169 relative parasite abundance in naturally infected macaques with a wide range of parasite burdens.

170 <u>Serial deep-sampling PCR can reveal changes in parasite burden over time</u>

171 In general, the frequency of positive PCRs and the Cq values of those positive reactions were relatively
172 stable in macaques across the 1yr study period. The major exceptions to this rule are shown in 3c and
173 3d. The three macaques in 3c had among the highest fraction of positive PCR reactions (70, 81, and 68
174 %) in the study, but also displayed an increase in mean Cq values over the 1yr study period. Because of
175 the consistent detection of positive PCR reactions (minimum 1 positive reaction in a maximum of 20
176 replicates every month) in 9 of the 18 initially sampled seropositive macaques (Figure 1), these
177 macaques were only sampled for the 1st 6 months of the study period. However, for these macaques in
178 Fig 3c with a pattern of increasing Cq values over 6 months, an additional set of samples was collected at
179 month 12. In all three cases, a highly significant ($P<0.001$ in all cases) trend of increasing Cq values over
180 time was evident, indicating a decreasing parasite load over time. Not surprisingly, these three
181 macaques were also among the most recently infected macaques studied (minimum 2-4 years post-
182 infection) and likewise, the macaques in 3a with a low frequency of PCR positive reactions were among
183 the longest infected of the macaques under study (6-8 years). However, several macaques did not match
184 this trend of lower parasite load with increasing length of infection (e.g. P11, infected for 2 years, had a
185 low parasite load and P5 and P1, infected respectively for 8 and 10 years, had among the highest
186 frequency of PCR positive reactions). There was no statistically significant correlation between the
187 length of infection and the frequency of PCR positive reactions ($p= 0.081$) or the mean Cq value of
188 positive PCR reactions ($p= 0.11$) among all macaques in the study (Figure 3e). This monthly monitoring of
189 blood parasite DNA thus reveals a relatively stable parasite load over 12 months but huge differences
190 when compared to the spiked samples (Figure 1) in the load between individual naturally infected
191 macaques, irrespective of the length of infection.

192 The other exception to a relatively stable level of *T. cruzi* DNA in blood of infected macaques is shown in
193 Figure 3d. Macaque P4 was PCR negative on 384 replicate reactions in the first month of sampling, but
194 became strongly positive with 50% of the 20 replicate reactions in months 2 and 3 of the study,

195 indicating a rapid and dramatic change in *T. cruzi* DNA in blood. This animal developed uncontrolled
196 diarrhea late in the 3rd trimester of pregnancy and had to be euthanized shortly after collection of the
197 August sample. Necropsy and histopathology revealed chronic typhlocolitis with locally extensive
198 ulceration at the ileocecal junction suggestive of a pre-neoplastic or early neoplastic lesion, as well as
199 mild cardiac fibrosis with minimal lymphohistiocytic infiltrate. The origins of the loss of immune control
200 of *T. cruzi* infection in this animal were not further explored, but this episode emphasizes that a well-
201 contained chronic *T. cruzi* infection can rapidly yield to a high-level/uncontrolled infection.

202 Lastly, with the exception of a set of samples that were cross-contaminated during DNA isolation in the
203 February batch (see Materials and Methods), and a single replicate PCR for N1, the two seronegative
204 macaques as well as 5 macaques previously infected but cured by treatment with benzoxaborole
205 AN15368 [8] were PCR negative in a collective 15,084 PCR reactions on samples obtained at 6 to 11
206 sampling points (S1 Fig.) and were likewise hemoculture negative in all samples.

207 <u>DNA fragmentation further increases the sensitivity of detection of *T. cruzi* DNA in blood</u>

208 We noted that with the input of 10^{-3} PE per replicate PCR reaction, which consistently gives 100%
209 positive reactions (Fig 1), that these positive reactions were mostly clustered at a relatively low Cq value.
210 However, decreasing the amount of spiked-in parasite DNA to 10^{-4} PE or lower resulted in a wide spread

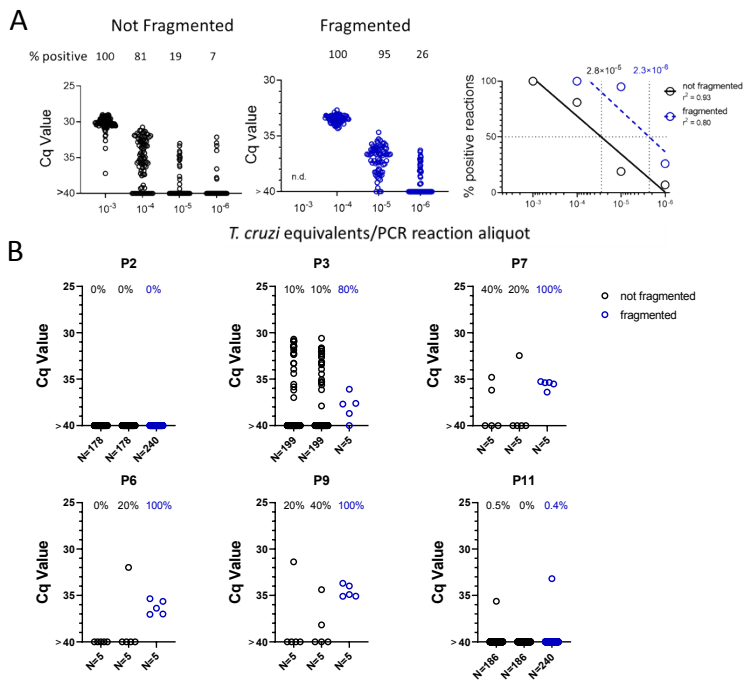


Figure 4. DNA Fragmentation increases the sensitivity of PCR detection of *T. cruzi* DNA. A) The quantity of parasite DNA required to yield 50% positive PCR reactions in replicate assays is reduced by >10-fold by cuphorn fragmentation of DNA from blood. A total of 72 to 96 replicate PCR reactions were conducted for each sample. B) The increased sensitivity of consistent detection of *T. cruzi* DNA achieved by prior fragmentation is evident in samples from infected macaques. Blood samples were collected as described in Figure 2A for samples A and B but in this case, sample C was also used for DNA isolation and that DNA fragmented by cuphorn sonication. N=the number of replicate PCR

211 in the Cq values, in addition to increasing frequency of negative reactions with decreasing PE/reaction.
212 The parasite DNA sequence targeted for amplification in these assays is a "satellite DNA" sequence that
213 is represented in 100,000 or more copies spread throughout the parasite [10]. We reasoned that the
214 spread of Cq values at low DNA input might reflect an unequal distribution of linked PCR target
215 sequences in the aliquots and that fragmentation of the DNA could more evenly distribute the target
216 sequences throughout the blood DNA sample, and thus increase the frequency of positive PCR
217 reactions. Comparison of fragmented and non-fragmented parasite-spiked blood DNA samples
218 confirmed this hypothesis, shifting the 100% positive PCR reaction one order of magnitude more
219 sensitive (from 10^{-3} to 10^{-4} PE per reaction; Figure 4a). Fragmentation by either Covaris ultrasonication
220 or a cuphorn attachment to a standard sonicator proved equally effective in increasing assay sensitivity
221 (S2 Fig).

222 To determine if DNA fragmentation also increased the sensitivity of detection of *T. cruzi* DNA in blood
223 samples from infected hosts, we resampled 6 macaques, focusing on those that had been the most
224 difficult to detect in the initial 1-yr sampling study (Figure 3 and S1 Fig.). Samples were collected and
225 processed similarly as shown in Figure 2a except that "C" samples were processed for DNA extraction
226 and then fragmented and compared to the unfragmented samples A and B. As with the DNA-spiked
227 samples, prior DNA fragmentation increased the sensitivity of PCR detection; fragmented samples from
228 4 of the 6 macaques had 80-100% positive reactions using only 5 replicates, indicating that a single PCR
229 reaction could have detected most of these as *T. cruzi* positive while the unfragmented samples had
230 frequencies of positive amplification from 0 to 40% (Figure 4b). Fragmentation was also beneficial in
231 animals with higher parasite loads, providing 100% positive PCRs when fragmented compared to as low
232 as 20% in the same nonfragmented samples (S3 Fig.).

233 <u>Replicate sampling, but not time between samples, facilitates detection of *T. cruzi* infection in hosts with</u>
234 <u>the lowest parasite burden</u>

235 Altogether, these results demonstrate that deep-sampling PCR can consistently detect *T. cruzi* DNA in
236 the blood of *T. cruzi*-infected macaques well below the 10^{-3} level (equivalent to ~0.5 parasites/ml of
237 blood) typically considered the limit of quantification in *T. cruzi* PCRs [2] and that prior fragmentation
238 can reduce by 10X the number of replicate reactions needed to detect a positive. However, animals
239 with low and/or infrequent circulating parasite DNA still resist detection at a single bleed point (e.g.
240 Figure 4; macaques P2 and P11); indeed, a number of macaques were negative by deep sampling PCR on
241 duplicate blood samples at greater than half of the monthly sampling points. To determine if this
242 variation in detection at different sampling times was random (due primarily to sampling error) or
243 reflected true fluctuations in parasite load over time, the six macaques shown in Figure 4 were sampled
244 an additional 7 times over 4 weeks, and replicate fragmented blood DNA samples submitted to deep-
245 sampling PCR.

246 As shown in Fig 5a, blood sampling over this short time frame gave a similar irregular pattern of DNA
247 detection as did the monthly samples, with some animals >80% positive for replicate PCRs at some
248 points and 0% positive on multiple others (e.g. P2 and P7). It was also striking that in multiple animals,
249 relatively high PCR positive rates were observed in one of the samples but not in the duplicate sample
250 collected at the same time (indeed usually using the same needle stick; e.g. P2 4; P9 bleeds 5 and 8; and
251 P11 bleed 8). These results emphasize the random sampling error involved in detecting *T. cruzi* DNA in
252 hosts with very low parasite burden. However, collectively, the overall frequency of positive PCR

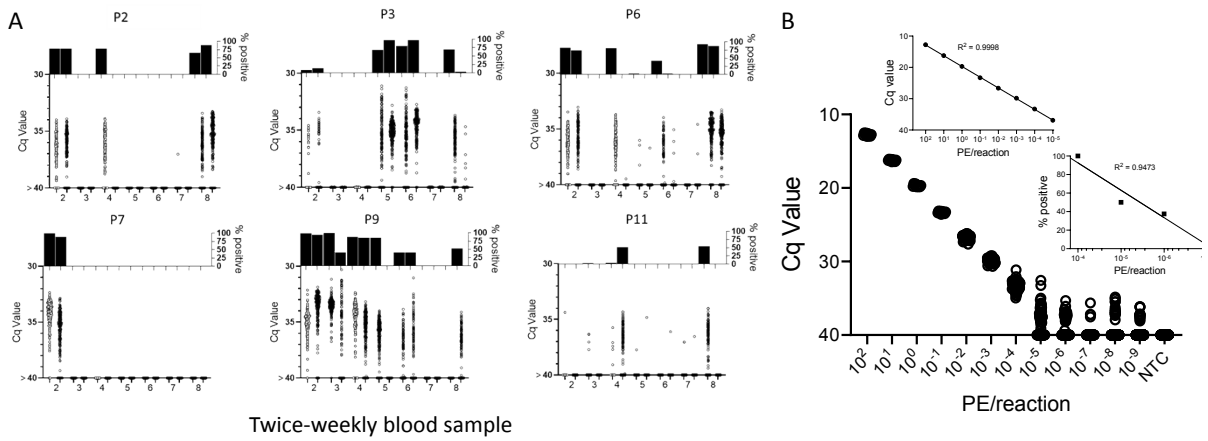


Figure 5. Repeat blood sample collection and deep-sampling PCR of fragmented DNA can quantify infection load over a minimum of 8 orders of magnitude. A) Frequent blood sampling demonstrates the sampling error involved in the detection of *T. cruzi* DNA in infected macaques with low parasite burden. Duplicate blood samples were collected twice weekly for 4 weeks from six macaques with the lowest overall PCR positive rate in the monthly sampling study (Figure 3 and S1 Fig). Bleed 1 was used in the experiments in Figure 4; the results of bleeds 2-8 are shown here. DNA was extracted from the duplicate samples at each bleed point and subjected to fragmentation before aliquots were used in 184 replicate PCR reactions/sample. The bottom of each subfigure shows the Cq value of each replicate reaction and the top plots the % positive reactions. B) Replicate PCR analysis of fragmented macaque blood DNA spiked with known parasite equivalents (PE) of *T. cruzi* DNA. Insets show the linear relationship between Cq values and PE/reaction over the high range of PE and percent positive reactions and PE/reaction on the lower range of inputs. 10 to 388 replicate reactions were conducted for each dilution. NTC = no *T. cruzi* DNA

253 reactions for these 6 macaques in year 2 (8, twice weekly collections; fragmented DNA) compared to the
254 same animals in year 1 (11-12 monthly collections; non-fragmented DNA; S1 Table) was >10-fold higher
255 (2.2% vs 27.5%).

256 To determine the full range of quantification of *T. cruzi* DNA in blood that is possible using both DNA
257 fragmentation and deep sampling, we analyzed blood spiked with *T. cruzi* DNA over 12 orders of
258 magnitude (Fig 5b). As noted above, DNA fragmentation alone extends the range over which a single
259 PCR reaction is dependably positive to 10^{-4} PE/reaction. Below 10^{-4} PE/aliquot, both the average Cq
260 value and the percent positive reactions allow quantification to 10^{-5} PE and the percent positive
261 reactions remains linear to 10^{-7} PE/assay. Thus, the combination of fragmentation and deep-sampling
262 extends the quantifiable range of detection of *T. cruzi* by 4 orders of magnitude below previous
263 estimates, to ~2.4 x 10^{-5} parasites/ml of blood, or fractions of a parasite in blood DNA. This detection
264 level is also consistent with the known frequency of the target satellite DNA repeat (approximately
265 10^5/parasite) and suggests that the assay is approaching single copy DNA detection/reaction. Detection
266 of *T. cruzi* is possible well below this limit of quantification but is essentially random and is dependent
267 on the number of blood samples and their volume collected, as well as the number of replicate PCR
268 reactions conducted on each.

269 The blood cell pellet routinely contains the highest amount of detectable parasite DNA relative to other
270 blood fractions

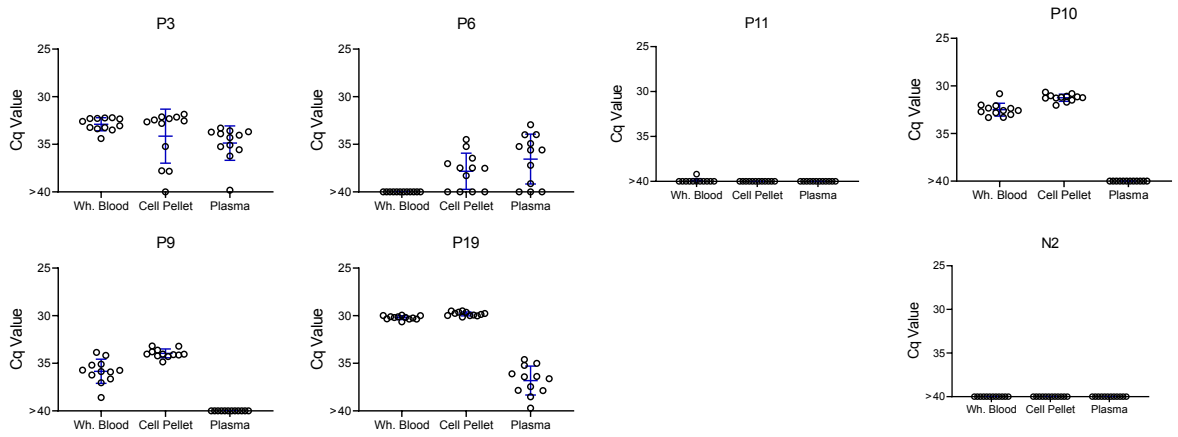


Figure 6. *T. cruzi* DNA can be detected in whole blood, the cell pellet of heparinzed blood, or plasma, but is at the highest and most consistently detected in the blood cell pellet. Replicate blood samples taken at the same time were either processed as whole blood (sample 1) or separated into the cell pellet and plasma (sample 2) by centrifugation, for DNA purification and fragmentation. Twelve replicate aliquots of 125 ng of DNA each were amplified by PCR for each fraction. Mean and standard deviation are shown.

271 Several recent studies have successfully used patient serum or plasma as a source for detection of *T.*
272 *cruzi* DNA [11, 12]. To directly determine the optimal source of DNA for parasite detection, we
273 compared DNA extracted from whole blood, the cell pellet from blood, or plasma. In all cases, 125 ng of
274 the source DNA was used for each replicate PCR. Although all DNA sources provided positive replicates
275 for one or more animals, both plasma DNA and whole blood DNA had all negative replicates in several
276 cases when the replicates of DNA from the cell pellet were majority positive (Fig 6). Thus, plasma is a
277 useful, but probably less dependable source for detection of *T. cruzi* DNA in the blood of infected
278 macaques.

279 Deep-sampling PCR demonstrates that humans and dogs exhibit a similar range of *T. cruzi* burdens as
280 macaques and detection of DNA in blood predicts DNA detection in tissues

281 The deep sampling approach optimized using samples from naturally infected macaques performs
282 similarly with blood samples from humans and dogs (Figure 7) and both species exhibited the same
283 broad range of detectable parasite DNA in circulation across individuals as observed in the macaques.
284 Although it is rare to be able to compare parasite detection in blood or plasma with that in tissues of the
285 same animal, we had that opportunity in three *T. cruzi*-infected animals, a dog which succumbed to
286 heartworm infection, and two macaques who were euthanized for health reasons unrelated to *T. cruzi*
287 infection. As expected, animals with detectable blood parasite DNA also had PCR-amplifiable DNA in
288 samples from multiple tissues (S4 Fig.).

289 Lastly, we used the deep sampling PCR approach to monitor outcomes in dogs under treatment using
290 benznidazole in a high-dose/less frequent dosing protocol which has shown variable efficacy in multiple

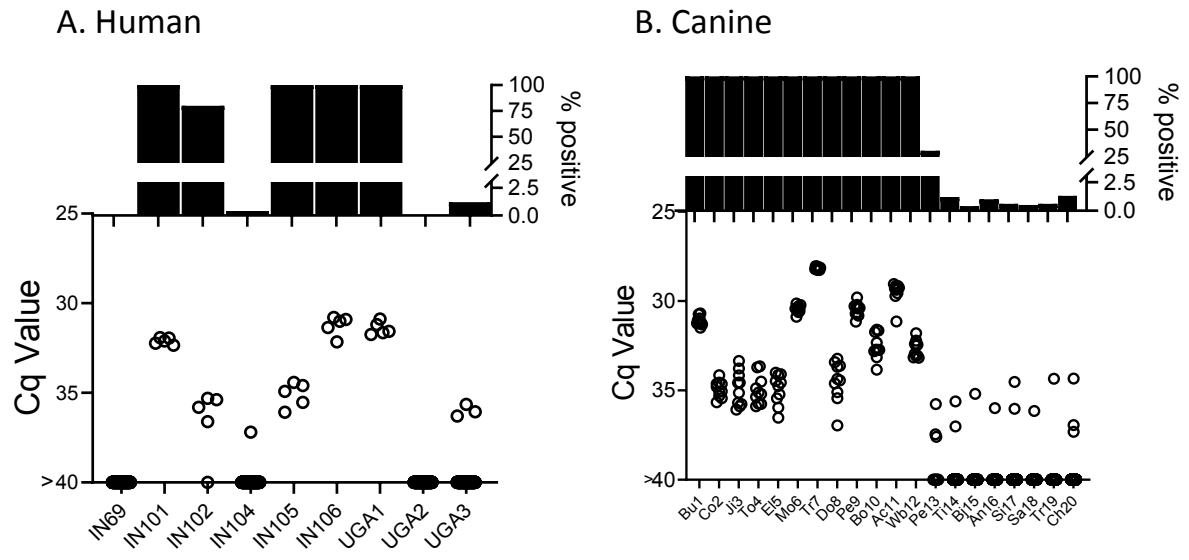


Figure 7. Deep sampling PCR of A) fragmented DNA from whole blood from 9 not-treated, chronically infected humans and B) fragmented blood cell pellet DNA from 20 seropositive dogs. An additional 10 seropositive dogs from this study group were negative for up to 384 replicate PCR reactions (not shown).

species [13, 14]. Treatment monitoring by deep-sampling PCR in 3 dogs with different treatment outcomes is shown in Fig 8. TFu1 was infected as a puppy and one of his littermates succumbed to *T. cruzi* infection before treatment could be initiated [15]. Twice weekly dosing for nearly 12 months reduced, but did not totally clear parasites from blood. Increasing the dosing frequency to 3 times per week resulted in undetectable parasites. Twice weekly dosing in TDa2 also initially appeared to be successful but detection of *T. cruzi* DNA in samples collected at 10 and 28 weeks prompted a decision to change the dosing frequency to 3 times per week. TPe3 still had robust levels of *T. cruzi* DNA at one week post-treatment but was negative by deep-sampling PCR while on a twice weekly dosing regimen.

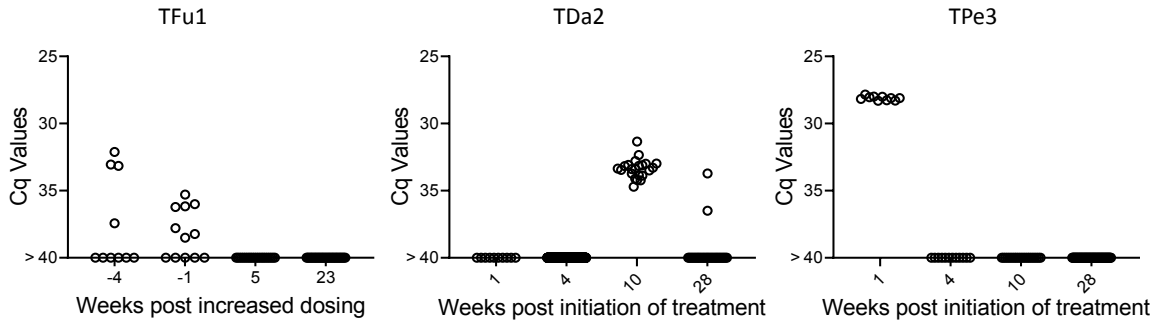


Figure 8. Monitoring by deep sampling PCR of *T. cruzi* DNA from whole blood in dogs during treatment with benznidazole. Dogs were treated with 18-20 mg/kg benznidazole twice (initially) or thrice (if remaining PCR positive) per week.

299 Proof of cure in these dogs will require evidence of continued negative deep-sampling PCR after the end
300 of treatment, but these results demonstrate that, as expected based upon many studies in humans, the
301 response to BNZ treatment can be variable between individuals but that rigorous assessment of
302 treatment effectiveness during the treatment course allows adjustments to dosing that may provide
303 higher overall treatment success.

304 **Discussion**

305 Without question, the inability to detect and quantify *T. cruzi* in blood or other samples from individuals
306 with suspected infection, is **the** key impediment to identifying candidates for treatment, determining if
307 the treatment has been effective in resolving the infection, and validating potential new treatments.
308 Additionally, without the ability to routinely quantify parasite load in humans and other naturally
309 infected hosts, there are multiple, sometimes contentious issues in Chagas disease that will remain
310 unresolved, among these, the relative role of parasite and host genetics in establishing the parasite
311 burden in this persistent infection, the stability of parasite load over time, and particularly important,
312 the relationship between parasite burden and the presence and severity of clinical disease.

313 Quantifying *T. cruzi* in blood and other tissues in hosts after the early peak of infection is challenging
314 because the immune response to *T. cruzi* infection is normally extremely effective in tightly limiting
315 parasite numbers within a few months of the initial infection. The bulk of *T. cruzi* in vertebrate hosts at
316 any point in time are intracellular, mostly in various muscle types throughout the body. However, to
317 maintain and to transmit the infection, *T. cruzi* must also exit host cells (following a 4-5 day period of
318 parasite multiplication) and infect new host cells or on occasion, be ingested by blood-feeding
319 triatomines. In addition to intact parasites, the DNA released by parasites that are killed by immune
320 effector mechanisms may also enter the bloodstream and thus be available for detection, either free in
321 plasma or bound to blood cells [16, 17]. In short, even in hosts with very low-level/well-controlled
322 infections, the potential always exists for *T. cruzi* DNA to be present in the blood, albeit often at
323 extremely low amounts. And when present in such low quantities, the chances of detection, even with
324 highly optimized PCR protocols, is random and highly prone to sampling error. This is not a situation
325 unique to *T. cruzi* infection. For example, detection of SARS-CoV-2 [18] and HIV [19] using PCR is also
326 prone to failure when employing single amplification reactions. The ability of replicate PCR reactions to
327 overcome this limitation has been discussed in depth in PCR optimization reviews [20] and Stowers et al.
328 [21] reported that by averaging hundreds of replicate reactions one can vastly extend the concentration
329 range over which PCR can provide reliable detection of DNA that is in low abundance. However, the use
330 of hundreds of replicate PCR reactions as conducted here has been rarely, if ever, used to routinely
331 validate the presence of any infectious agent in blood.

332 It is well-documented that *T. cruzi*-positive hosts give variable results in PCR tests, and this variability
333 increases with decreasing parasites or parasite DNA in a sample. This is primarily due to subsampling
334 error [20] and this error functions at two levels when screening blood for the presence of pathogen
335 DNA. First, a blood sample is a subsample of the entire blood volume of a potential host; e.g. a 10 ml
336 blood sample is ~0.2 percent of the total volume of blood in an average adult human. The existence of
337 this subsampling error in *T. cruzi* infection is well-illustrated by the fact that two replicate blood samples
338 from macaques collected using the same needle stick can yield a substantial number of positive PCR
339 reactions in one sample, and zero positive reactions in the other (Figure 5). Simply collecting samples at
340 different times, whether a few days (Figure 5) or a few months (Figure 3) apart, does not necessarily

341 solve this problem. When parasite numbers are very low, the chance that a small subsample of the total
342 blood will contain parasite DNA is random irrespective of when it is collected. The only solution is to
343 obtain more or larger blood samples so as to increase the chances of collecting a fragment of the
344 parasite DNA that is in the circulation. This same subsampling error acting at the level of whole parasites
345 was evident in the xenodiagnosis studies of Cerisola, wherein it was rare to obtain parasite-positive bugs
346 when they fed on certain subjects who apparently had very low levels of parasites circulating in the
347 blood [6].

348 A second opportunity for subsampling error under low target conditions occurs when an aliquot of the
349 total blood DNA obtained in a blood draw is used for PCR amplification. In the case of the ~5 ml
350 macaque blood samples used in the bulk of the assays in this study, from 1/100th to 1/250th of the total
351 recovered DNA was used for each replicate PCR reaction. In a number of animals, the entire blood DNA
352 sample was exhausted doing replication PCR reactions without a positive reaction while in most animals,
353 some DNA remained after nearly 200, sometimes all negative, amplification reactions/sample. We show
354 that fragmentation of DNA significantly decreases the subsampling error at this level, presumably by
355 breaking the DNA containing one or more target satellite DNA regions into smaller fragments that are
356 then more widely dispersed in the total blood DNA. DNA fragmentation led to an increased frequency
357 of positive replicate PCR reactions and generally more consistent Cq values between these aliquots.
358 Fragmentation increased amplification consistency and the sensitivity of the PCR protocol by one order
359 of magnitude and should be incorporated as a standard step for amplification of satellite DNA target in
360 *T. cruzi* even when deep-sampling is not used. However ultimately, the limiting factor in the consistent
361 detection of *T. cruzi* in blood is the total amount of blood collected and processed for fragmentation and
362 amplification. If there is no parasite DNA in a particular sample, no amount of fragmentation or number
363 of amplification reactions will detect it. Also, in addition to sampling errors, the inefficiency of PCR to
364 amplify very low abundance targets, particularly among a complex DNA mixture as exists in blood
365 (termed the Monte Carlo effect [22, 23]) may also may contribute to the failure to detect target *T. cruzi*
366 DNA in some samples despite its presence.

367 In addition to providing much greater sensitivity for detecting active infection relative to conventional
368 single or low replicate (2-3/sample) PCR analysis, conducting 100's of replicate PCR reactions on
369 fragmented blood DNA also provides information on the relative abundance of parasites in the blood of
370 infected hosts. Most implementations of PCR for *T. cruzi* have a limit of consistent detection of ~10^{-3}
371 PE/assay, which equates to ~0.5 parasites/ml of blood in our assays. Combining sample DNA
372 fragmentation and deep-sampling extends the range of quantification to at least 10^{-6} PE per aliquot, or
373 ~0.00025 parasites/ml (1 parasite per 4 liters) of blood.

374 This considerably increased sensitivity of infection detection and relative quantitation made possible
375 through the use of deep sampling PCR reveals the previously undocumented range of parasite burden
376 between individuals with chronic *T. cruzi* infections. Although best demonstrated in the frequently
377 sampled macaques in this work, both humans and dogs also exhibit a range of parasite burdens
378 exceeding 5 orders of magnitude when compared to a standard curve (e.g. from Cq values of ~25 in the
379 highest macaque and dog fragmented samples to <1% frequency of positive PCR reactions in some
380 members of all three species). The similarity in the ranges of parasite load in the three species
381 examined in this study again reinforces the similarities between *T. cruzi* infection in these species and
382 the appropriateness of dogs and NHPs as models of the human infection [24].

383 In addition to the sampling error discussed above, the variation in the number of satellite DNA repeats
384 per genome among *T. cruzi* isolates [2] makes absolute quantitation of parasite load virtually impossible.
385 However, such quantitation is not necessary in order to conclude that individuals with long-established
386 *T. cruzi* infections control these infections to vastly different degrees. This ability to more precisely
387 quantify parasite load should now allow investigations into the potential immune mechanisms that
388 might be responsible for this variable control and assessment of the potential correlation between
389 relatively stable parasite load and the chances of having or developing clinical disease – which is
390 detected in <50% of long-term infected subjects.

391 This study also confirms across a broader range of individuals, the relative stability of parasite load over
392 time, an expected phenomenon that was also apparent in the xenodiagnosis studies by Cerisola [6]. A
393 surprising finding was that a subset of macaques infected for 2-3 years and presenting a relatively higher
394 parasite burden exhibited a significant decline in parasite load over the one-year survey period. This
395 result suggests that immune control mechanisms not only continue to confine parasite load but can also
396 drive that load lower over time during the chronic phase of infection. However, this is not the pattern in
397 all individuals, as some macaques with equally short-term infections have already restricted parasite
398 numbers to nearly undetectable levels while some with longer-term infections maintain relatively higher
399 parasite burden. It is also noteworthy that parasite control can be quickly lost when the health status
400 (and presumably immune status) changes, as in the case of macaque P4, similar to what is observed in
401 humans with suppressed immunity [25]. These findings emphasize that even subjects controlling *T. cruzi*
402 burden to very low levels should be considered for anti-parasite treatment as the immune control of the
403 infection can be quickly lost.

404 The original goal of this study was to develop a 'test of cure' for use in the evaluation of clinical trials of
405 anti-*T. cruzi* compounds. Previous candidate compounds used in clinical trials have failed to provide
406 sterile cure and these failures were relatively easy to detect without a highly sensitive PCR protocol as
407 described here ([26, 27]. With the progression of highly promising new compounds, including one that
408 provided 100% sterile cure in NHPs with naturally acquired *T. cruzi* infection [8], an assay that detects
409 success rather than only failure is needed. The combination of DNA fragmentation and deep-sampling
410 of replicate blood samples should fulfill that need. Operationally, this would not require any special
411 equipment but does necessitate extreme care in sample preparation and assay execution. Also,
412 conducting hundreds of PCR reactions on the DNA from 10 or more high volume (e.g. 10 ml or greater)
413 blood samples from each subject will be expensive, time-consuming and labor-intensive. It is also
414 worthy of note that in cases of treatment failure, the apparent abundance of *T. cruzi* DNA in the blood
415 often return to pre-treatment levels [5], so selection of subjects with more readily detectable pre-
416 treatment parasite loads for such trials would make it relatively easy to detect treatment failures and
417 provide greater confidence that those that remain negative following deep-sampling are indeed cured.

418 There may also be opportunities for additional improvements in PCR-based detection of *T. cruzi*. One of
419 the limitations of this study is that we have so far only examined samples from a small number of
420 human subjects. However, those samples fall into the same pattern of wide-ranging parasite loads as
421 the macaque and dog samples so there is little reason to think that more extensive testing of human
422 samples will reveal any surprises. Additional targets for PCR amplification have been identified [11, 12]
423 and could be multiplexed in a single reaction to achieve greater sensitivity. We also may not have
424 exhausted the limits of DNA fragmentation for dispersing target DNA in a blood sample in order to
425 further reduce subsampling errors or explored the tolerance for increased loading of fragmented DNA

426 for each PCR reaction. Improvements in any of these areas could reduce the number of replicate PCR
427 reactions without compromising sensitivity. Finally, new technologies such as UltraPCR, which allows
428 for higher DNA loading, extensive multiplexing, and the generation of >30 million individual PCR reaction
429 per tube ([28, 29] could greatly reduce the number of replicate PCR assays that are needed for high
430 sensitivity detection. Such developments could make the PCR-based detection of all *T. cruzi*-infected
431 subjects possible, if not routine.

432 **Materials and Methods**

433 Macaques

434 All NHP utilized for these studies were part of the approximately 1000-animal, Rhesus Macaque
435 (*Macaca mulatta*) Breeding and Research Resource housed at the AAALAC accredited, Michale E.
436 Keeling Center for Comparative Medicine and Research (KCCMR) of The University of Texas MD
437 Anderson Cancer Center in Bastrop. TX. This is a closed colony, which is specific pathogen free (SPF) for
438 Macacine herpesvirus-1 (Herpes B), Simian retroviruses (SRV-1, SRV-2, SIV, and STLV-1), and
439 *Mycobacterium tuberculosis* complex. Study animals that were seropositive for *T. cruzi* had acquired the
440 infection naturally through exposure to the insect vector of the parasite while in their indoor-outdoor
441 housing facilities. The NHP experiments were performed at the KCCMR and all protocols were approved
442 by the MD Anderson Cancer Center's IACUC (ACUF# 00002241-RN00 and 00000451-RN03), and followed
443 the NIH standards established by the Guide for the Care and Use of Laboratory Animals [30].

444 A total of 26 rhesus macaques that had been confirmed to be serologically positive for *T. cruzi* infection
445 (a subset of which had been PCR positive in previous screenings ([7]; S1 Table), were utilized in these
446 studies. Five of these macaques were previously treated and cured of *T. cruzi* infection in 2018 and
447 consistently PCR negative since [8]. These cured macaques and two seronegative macaques served as
448 control, uninfected animals. Under light injectable anesthesia, three ~5 ml peripheral blood samples
449 (total ~15 ml) were collected from each animal and shipped overnight on ice packs for each sampling
450 point. Except as indicated, whole blood was used for DNA extraction.

451 Canines

452 Dogs used in these studies came from a network of kennels in central and south Texas with a history of
453 triatomine vector occurrence and canine Chagas disease as previously described [31]. At these large
454 kennels, dogs are primarily bred and trained to aid hunting parties and the predominant breeds include
455 Belgian Malinois, Brittany spaniels, cocker spaniels, English pointers, German shorthaired pointers,
456 Kelpies, Labrador retrievers, and hound dogs. Dogs >2 months of age, including males and females were
457 sampled. Approximately 3ml of blood was collected via jugular venipuncture into heparinized tubes
458 which were centrifuged at 2,000 g for 15 minutes and the cell pellet and plasma separated before
459 overnight shipment on ice. *T. cruzi* seropositive dogs were identified by *T. cruzi* multiplex serology [31]
460 before blood cell pellet DNA testing by PCR. Some dogs previously confirmed as infected were treated
461 using a twice-weekly high-dosing protocol as previously described [13].

462 Informed consent was obtained from dog owners prior to their participation, and this study was
463 approved by the Texas A&M University Institutional Committee on Animal Use and Care and the Clinical
464 Research Review Committee (IACUC 2018-0460 CA and IACUC 2022-0001 CA).

465 Human

466 Nine subjects with positive serological findings for *T. cruzi* infection (ie, positive in ≥ 2 of the 3 tests
467 performed, indirect immunofluorescence assay, hemagglutination, and enzyme-linked immunosorbent
468 assay; as previously described [32]) were enrolled at the Hospital Interzonal General de Agudos Eva
469 Perón, in Buenos Aires, Argentina. All participants had no signs of cardiac disease as revealed by
470 electrocardiography and echocardiography testing. The protocol was approved by the Institutional
471 Review Board (IRB) of Hospital Interzonal General de Agudos Eva Perón (Memorandum 19/19) of the
472 Province of Buenos Aires, Argentina. Signed informed consent was obtained from all individuals included
473 in the study. Ten milliliters of blood were drawn from seropositive subjects by venipuncture into
474 heparinized tubes (Vacutainer; BD Biosciences) and centrifuged at 1000 × g for 15 min. The plasma was
475 collected and the blood clot resuspended and both samples frozen at – 20°C and shipped to the
476 University of Georgia on dry ice.

477 Blood DNA extraction and qPCR

478 DNA was extracted from all blood samples or fractions thereof (whole blood, pellet, plasma layer) using
479 the Omega E.Z.N.A Blood DNA MAXI kit. Following the manufacturers protocol for "up to 10ml Whole
480 blood", approximately 2-5 mL of blood or blood fraction was lysed and processed bringing the initial
481 volume of each sample up to 10 mL with the addition of PBS (Gibco 10010023) and eluting with 500 µl
482 elution buffer. Samples that were frozen upon receipt (human samples) were thawed on ice before
483 starting protocol. DNA samples were quantified with the nanodrop 2000 system (Themo Scientific)
484 before diluting to 25 ng/µl in water (Invitrogen AM9937).

485 The qPCR assay used to detect *T. cruzi* DNA in blood in this study is essentially as previously described
486 [8] and used extensively in many labs, including those involved in clinical trial monitoring [3]. In brief,
487 each reaction includes 125 ng of genomic DNA, 1 pg of internal amplification control (IAC) fragment,
488 0.75 µM of each *T. cruzi* satellite DNA-specific primer and IAC-specific primers, 0.5µM each of *T. cruzi*
489 satellite DNA and IAC probes and 10 µl of BioRad iTaq Universal Probes Supermix and water to make a
490 20µl final volume reaction. IAC template, primer and probes are omitted from deep-sampling assays
491 following the first 5-10 replicates. We used 384-well hard-shell plates (Bio-Rad # HSP3805) and
492 Microseal® 'B' Adhesive Seals (Bio-Rad #MSB1001) compatible with qPCR assays and the CFX Opus 384
493 real time PCR detection system under the following cycling conditions: (i) initial denaturation, 95°, 3
494 min; (ii) denaturation, 95°C, 15 s; (iii) annealing, 58°C, 1 min; (iv)×50 cycles. A standard sample with a
495 known concentration of T. cruzi DNA 5.6 $x10^{-3}$ is included in each plate for reference. Analysis of the
496 data was done using CFX Maestro software version 2.3 (Bio-Rad). IV.

497 Preventing contamination

498 To prevent contamination when preparing high numbers of qPCR reactions, a number of safeguards
499 were employed. The setup for all reactions was performed in a space separate from our main research
500 laboratory and in a class II A2 laminar flow hood otherwise not used for work with *T. cruzi* and using
501 supplies and instruments that were specific for this lab space. All components are sprayed with 70%
502 ethanol and DNA decontamination spray (LookOUT, Sigma) before being brought into the hood and all
503 hood surfaces are sprayed with ethanol and decontaminant at the end of each work session, followed
504 by UV exposure. PCR assay components were aliquoted from large batches into individual tubes
505 sufficient for each 384-well plate. The centrifuge used for DNA extractions was bleached and
506 decontaminated before processing the final elution for each DNA batch. DNA samples are diluted into
507 96 well plates and then loaded 96 wells at a time into the 384 well plate using the mini 96 pipette

508 (Integra Mini 96). Despite these procedures, one set of DNA isolations from *T. cruzi*-negative controls
509 showed evidence of cross contamination when they were all processed at the same time as a set of DNA
510 standards containing high quantities of *T. cruzi* DNA. These "February" samples for 7 animals were
511 excluded from further analysis. Otherwise, samples from animals previously cured of *T. cruzi* infection by
512 treatment with AN15368 [8] processed at the same time as other "infected" samples showed no
513 evidence of cross-contamination.

514 DNA fragmentation

515 Purified DNA from blood was fragmented by either in a Covaris E220 Focused-ultrasonicator (which
516 allowed precise selection of target DNA fragment sizes of 1000, 500 or 300 bases), or using the 3in
517 cuphorn attachment to a Branson SFX250 Sonifier cooled by a Bio Rad 1000 mini chiller circulator. For
518 cuphorn sonication, DNA samples (typically 500 µl in volume and 15 µg to 150 µg total DNA) in 1.5 ml
519 Eppendorf snap cap tubes were wrapped securely in parafilm and kept on ice before fragmentation.
520 Sonifier settings were set to continuous mode with a time interval of 50 seconds and 60% amplitude for
521 5 cycles.

522 PCR standards

523 Samples containing known amounts of parasite DNA were generated using 5 ml whole blood with 10^7 *T.*
524 *cruzi* epimastigotes of the Brazil strain added. The blood was then treated the same as experimental
525 blood with DNA extracted as described with the Omega MAXI kit. The DNA was diluted to 25 ng/µl and
526 serially diluted 10-fold with 25 ng/µl naïve blood DNA.

527 Hemoculture

528 Peripheral blood (~5 ml) was aliquoted into 5 replicate T25 flasks for incubation at 26°C in supplemented
529 liver digest neutralized tryptose medium as described previously [9]. The presence of *T. cruzi* parasites
530 was assessed every week for up to 3 months under an inverted microscope.

531 Tissue PCR

532 Single tissue samples (8mm biopsies) or pooled samples from 5 sites totaling ~500 µl per pool were
533 obtained from frozen necropsied tissues, processed and subjected to PCR amplification of *T. cruzi* DNA
534 as previously described [8].

535 **Acknowledgements**

536 Special thanks to Stephanie Collins, MS, DVM for field collection of dog samples and Ty McAdams and
537 Luke Segura from KCCMR for assistance with coordination of NHP sampling. We thank the staff and
538 patients of the Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina who provided
539 blood samples.

540

541 **Supplementary Information Captions**

542 S1 Table. Historical and year one PCR and hemoculture summary for macaques

543 S1 Fig. The monthly pattern of detection of *T. cruzi* in replicate PCR reactions in DNA from macaque
544 blood collected over one year of sampling.

S2 Fig. Fragmentation of DNA increases the frequency of positive replicate PCR reactions in a DNA sample.

S3 Fig. DNA fragmentation results in more consistent PCR amplification and detection, even in samples where *T. cruzi* DNA may be detectable using a single PCR assay.

S4 Fig. Detection of *T. cruzi* infection by deep-sampling PCR of blood- or plasma-derived DNA is corroborated by the PCR detection of *T. cruzi* DNA in individual tissue samples from skeletal muscle, heart, or other organs including liver, spleen and gut.

References

1. Cucunuba ZM, Manne-Goehler JM, Diaz D, Nouvellet P, Bernal O, Marchiol A, et al. How universal is coverage and access to diagnosis and treatment for Chagas disease in Colombia? A health systems analysis. Soc Sci Med. 2017;175:187-98. Epub 2017/01/21. doi: 10.1016/j.socscimed.2017.01.002. PubMed PMID: 28107703.
2. Schijman AG, Bisio M, Orellana L, Sued M, Duffy T, Mejia Jaramillo AM, et al. International study to evaluate PCR methods for detection of Trypanosoma cruzi DNA in blood samples from Chagas disease patients. PLoS neglected tropical diseases. 2011;5(1):e931. Epub 2011/01/26. doi: 10.1371/journal.pntd.0000931. PubMed PMID: 21264349; PubMed Central PMCID: PMCPMC3019106.
3. Sulleiro E, Munoz-Calderon A, Schijman AG. Role of nucleic acid amplification assays in monitoring treatment response in chagas disease: Usefulness in clinical trials. Acta tropica. 2019;199:105120. Epub 2019/08/04. doi: 10.1016/j.actatropica.2019.105120. PubMed PMID: 31376368.
4. Silgado A, Moure Z, de Oliveira MT, Serre-Delcor N, Salvador F, Oliveira I, et al. Comparison of DNA extraction methods and real-time PCR assays for the molecular diagnostics of chronic Chagas disease. Future Microbiol. 2020;15:1139-45. Epub 2020/09/22. doi: 10.2217/fmb-2020-0023. PubMed PMID: 32954845.
5. Parrado R, Ramirez JC, de la Barra A, Alonso-Vega C, Juiz N, Ortiz L, et al. Usefulness of Serial Blood Sampling and PCR Replicates for Treatment Monitoring of Patients with Chronic Chagas Disease. Antimicrobial agents and chemotherapy. 2019;63(2). Epub 2018/12/05. doi: 10.1128/AAC.01191-18. PubMed PMID: 30509941; PubMed Central PMCID: PMCPMC6355557.
6. Cerisola JA. Chemotherapy of Chagas' Infection in Man. Chagas Disease Symposium Proceedings: PAHO; 1977.
7. Hodo CL, Wilkerson GK, Birkner EC, Gray SB, Hamer SA. Trypanosoma cruzi Transmission Among Captive Nonhuman Primates, Wildlife, and Vectors. Ecohealth. 2018;15(2):426-36. Epub 2018/03/03. doi: 10.1007/s10393-018-1318-5. PubMed PMID: 29497880; PubMed Central PMCID: PMCPMC6132415.
8. Padilla AM, Wang W, Akama T, Carter DS, Easom E, Freund Y, et al. Discovery of an orally active benzoxaborole prodrug effective in the treatment of Chagas disease in non-human primates. Nature Microbiology. 2022. doi: 10.1038/s41564-022-01211-y.
9. Padilla AM, Yao PY, Landry TJ, Cooley GM, Mahaney SM, Ribeiro I, et al. High variation in immune responses and parasite phenotypes in naturally acquired Trypanosoma cruzi infection in a captive non-human primate breeding colony in Texas, USA. PLoS neglected tropical diseases. 2021;15(3):e0009141. Epub 2021/04/01. doi: 10.1371/journal.pntd.0009141. PubMed PMID: 33788859; PubMed Central PMCID: PMCPMC8041201.
10. Ramirez JC, Cura CI, da Cruz Moreira O, Lages-Silva E, Juiz N, Velazquez E, et al. Analytical Validation of Quantitative Real-Time PCR Methods for Quantification of Trypanosoma cruzi DNA in

588 Blood Samples from Chagas Disease Patients. J Mol Diagn. 2015;17(5):605-15. Epub 2015/09/01. doi:
589 10.1016/j.jmoldx.2015.04.010. PubMed PMID: 26320872; PubMed Central PMCID: PMCPMC4698797.
590 11. Kann S, Kunz M, Hansen J, Sievertsen J, Crespo JJ, Loperena A, et al. Chagas Disease: Detection
591 of Trypanosoma cruzi by a New, High-Specific Real Time PCR. J Clin Med. 2020;9(5). Epub 2020/05/24.
592 doi: 10.3390/jcm9051517. PubMed PMID: 32443464; PubMed Central PMCID: PMCPMC7291166.
593 12. Kann S, Concha G, Weinreich F, Hahn A, Ruckert C, Kalinowski J, et al. Comparative Assessment
594 of Two Commercial Real-Time PCR Assays for the Diagnosis of Trypanosoma cruzi DNA in Serum.
595 Microorganisms. 2023;11(4). Epub 2023/04/28. doi: 10.3390/microorganisms11040901. PubMed PMID:
596 37110326; PubMed Central PMCID: PMCPMC10142699.
597 13. Bustamante JM, White BE, Wilkerson GK, Hodo CL, Auckland LD, Wang W, et al. Frequency
598 Variation and Dose Modification of Benznidazole Administration for the Treatment of Trypanosoma
599 cruzi Infection in Mice, Dogs, and Nonhuman Primates. Antimicrobial agents and chemotherapy.
600 2023;67(5):e0013223. Epub 2023/04/12. doi: 10.1128/aac.00132-23. PubMed PMID: 37039666;
601 PubMed Central PMCID: PMCPMC10190575.
602 14. Bustamante JM, Sanchez-Valdez F, Padilla AM, White B, Wang W, Tarleton RL. A modified drug
603 regimen clears active and dormant trypanosomes in mouse models of Chagas disease. Sci Transl Med.
604 2020;12(567). Epub 2020/10/30. doi: 10.1126/scitranslmed.abb7656. PubMed PMID: 33115952.
605 15. Lim S, Collins S, Hamer SA, Tarleton RL, Saunders AB. Positive clinical outcome using a modified
606 dosing regimen of benznidazole in dogs at high risk for infection or acutely infected with Trypanosoma
607 cruzi. J Vet Intern Med. 2024. Epub 2024/03/19. doi: 10.1111/jvim.17028. PubMed PMID: 38500407.
608 16. Lam LKM, Murphy S, Kokkinaki D, Venosa A, Sherrill-Mix S, Casu C, et al. DNA binding to TLR9
609 expressed by red blood cells promotes innate immune activation and anemia. Sci Transl Med.
610 2021;13(616):eabj1008. Epub 2021/10/21. doi: 10.1126/scitranslmed.abj1008. PubMed PMID:
611 34669439; PubMed Central PMCID: PMCPMC9065924.
612 17. Thompson JC, Li S, Jose JS, Predina J, Gupta A, Eruslanov E, et al. Red blood cells function as
613 reservoirs of tumor DNA. Am J Physiol Lung Cell Mol Physiol. 2024;326(5):L646-L50. Epub 2024/03/26.
614 doi: 10.1152/ajplung.00049.2024. PubMed PMID: 38529551.
615 18. Waked R, Makhoul J, Saliba G, Chehata N, Mortada S, Zoghbi A, et al. Are two consecutive
616 negative RT-PCR results enough to rule out COVID-19? New Microbes New Infect. 2020;37:100750. Epub
617 2020/09/03. doi: 10.1016/j.nmni.2020.100750. PubMed PMID: 32874594; PubMed Central PMCID:
618 PMCPMC7451052.
619 19. Rutsaert S, De Spiegelaere W, Van Hecke C, De Scheerder MA, Kiselinova M, Vervisch K, et al. In-
620 depth validation of total HIV-1 DNA assays for quantification of various HIV-1 subtypes. Scientific
621 reports. 2018;8(1):17274. Epub 2018/11/24. doi: 10.1038/s41598-018-35403-6. PubMed PMID:
622 30467426; PubMed Central PMCID: PMCPMC6250682.
623 20. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The Ultimate qPCR
624 Experiment: Producing Publication Quality, Reproducible Data the First Time. Trends Biotechnol.
625 2019;37(7):761-74. Epub 2019/01/19. doi: 10.1016/j.tibtech.2018.12.002. PubMed PMID: 30654913.
626 21. Stowers CC, Haselton FR, Boczko EM. An Analysis of Quantitative PCR Reliability Through
627 Replicates Using the C Method. J Biomed Sci Eng. 2010;3(5):459-69. Epub 2010/07/17. doi:
628 10.4236/jbise.2010.35064. PubMed PMID: 20634997; PubMed Central PMCID: PMCPMC2902203.
629 22. Karrer EE, Lincoln JE, Hogenhout S, Bennett AB, Bostock RM, Martineau B, et al. In situ isolation
630 of mRNA from individual plant cells: creation of cell-specific cDNA libraries. Proceedings of the National
631 Academy of Sciences of the United States of America. 1995;92(9):3814-8. Epub 1995/04/25. doi:
632 10.1073/pnas.92.9.3814. PubMed PMID: 7731989; PubMed Central PMCID: PMCPMC42052.
633 23. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain
634 reaction. J Biomol Tech. 2004;15(3):155-66. Epub 2004/08/28. PubMed PMID: 15331581; PubMed
635 Central PMCID: PMCPMC2291693.

24. Tarleton RLS, A. B.; Loccoco, B.; Alvarez Gianni, M.G.; Laucella, S., Hodo, C.L., Wilkerson, G.K.; Hamer, S.A. The Unfortunate Abundance of *Trypanosoma cruzi* in Naturally Infected Dogs and Monkeys Provides Unique Opportunities to Advance Solutions for Chagas Disease. Zoonoses. 2024. Epub 04 April 2024. doi: 10.15212/ZOONOSES-2024-0005.

25. Lattes R, Lasala MB. Chagas disease in the immunosuppressed patient. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2014;20(4):300-9. Epub 2014/03/08. doi: 10.1111/1469-0691.12585. PubMed PMID: 24602129.

26. Tarleton RL. Effective drug discovery in Chagas disease. Trends Parasitol. 2023;39(6):423-31. Epub 2023/04/07. doi: 10.1016/j.pt.2023.03.015. PubMed PMID: 37024318.

27. Tarleton RL. Avoiding clinical trial failures in neglected tropical diseases: The example of Chagas disease. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2022. Epub 2022/11/15. doi: 10.1093/cid/ciac884. PubMed PMID: 36373213.

28. Lai JH, Keum JW, Lee HG, Molaei M, Blair EJ, Li S, et al. New realm of precision multiplexing enabled by massively-parallel single molecule UltraPCR. bioRxiv. 2023. Epub 2023/10/24. doi: 10.1101/2023.10.09.561546. PubMed PMID: 37873291; PubMed Central PMCID: PMCPMC10592712.

29. Shum EY, Lai JH, Li S, Lee HG, Soliman J, Raol VK, et al. Next-Generation Digital Polymerase Chain Reaction: High-Dynamic-Range Single-Molecule DNA Counting via Ultrapartitioning. Anal Chem. 2022;94(51):17868-76. Epub 2022/12/13. doi: 10.1021/acs.analchem.2c03649. PubMed PMID: 36508568; PubMed Central PMCID: PMCPMC9798378 J.H.L., S.L., H.G.L., J.S., V.K.R., C.K.L., S.P.A.F., H.C.F. are employees of Enumerix, Inc., a company that commercializes DNA counting technologies. Enumerix, Inc., solely funded this study. In addition, E.Y.S., J.H.L., S.L., S.P.A.F., H.C.F. are inventors on patents and patent applications relating to methods described in this manuscript.

30. Animals NRCUCftUotGftCaUoL. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, DC: National Academies Press; 2011.

31. Bustamante JM, Padilla AM, White B, Auckland LD, Busselman RE, Collins S, et al. Prophylactic low-dose, bi-weekly benznidazole treatment fails to prevent Trypanosoma cruzi infection in dogs under intense transmission pressure. PLoS neglected tropical diseases. 2022;16(10):e0010688. Epub 2022/11/01. doi: 10.1371/journal.pntd.0010688. PubMed PMID: 36315597.

32. Castro Eiro MD, Natale MA, Alvarez MG, Shen H, Viotti R, Lococo B, et al. Reduced Trypanosoma cruzi-specific humoral response and enhanced T cell immunity after treatment interruption with benznidazole in chronic Chagas disease. The Journal of antimicrobial chemotherapy. 2021;76(6):1580-92. Epub 2021/03/12. doi: 10.1093/jac/dkab054. PubMed PMID: 33693664; PubMed Central PMCID: PMCPMC8600036.