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') 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 has been shown to 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).

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

Results

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

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

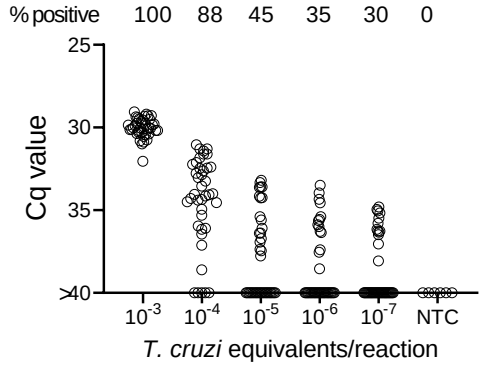

Figure 1. Deep-sampling (replicate PCR) allows detection of *T. cruzi* in a decreasing frequency of replicate reactions to at least 4 orders of magnitude below the normal limit of quantitation (10^{-3}) used for single PCR reactions. % positive is the percentage of replicate reactions that gave a detectable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

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

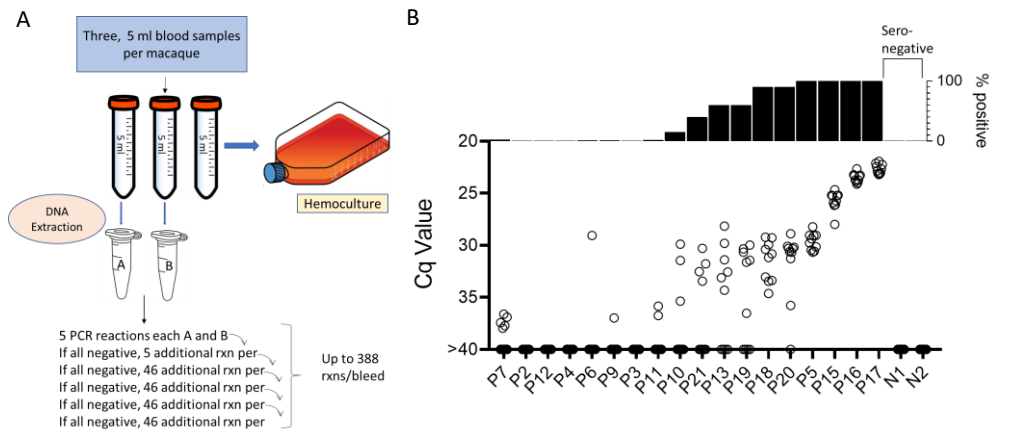

Figure 2. A. Protocol for the collection and PCR analysis of NHP blood samples. B. The combined PCR results of samples A and B at the first of monthly sampling points, plotting Cq values for all replicates (bottom) and percent positive replicates (top).

134

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

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

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

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

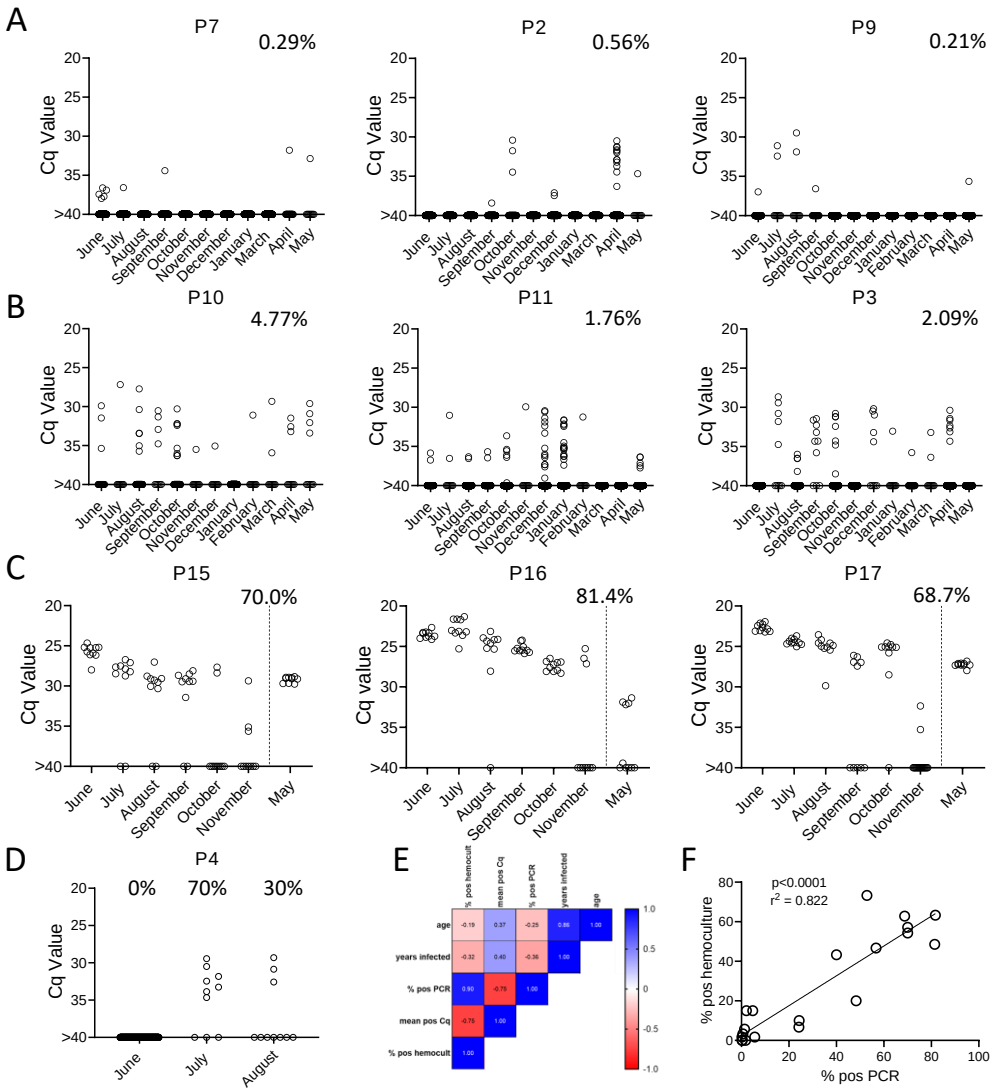

Figure 3. Monthly tally of representative macaques with A) rarely, B) variably or C) frequently positive PCR reactions over the one year of sampling. Animals in groups A) and B) were sampled all 12 months while those in C) were sampled only for the first 6 months then in the 12th month. Percentages indicate 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.

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

171 Serial deep-sampling PCR can reveal changes in parasite burden over time

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

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

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

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

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

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

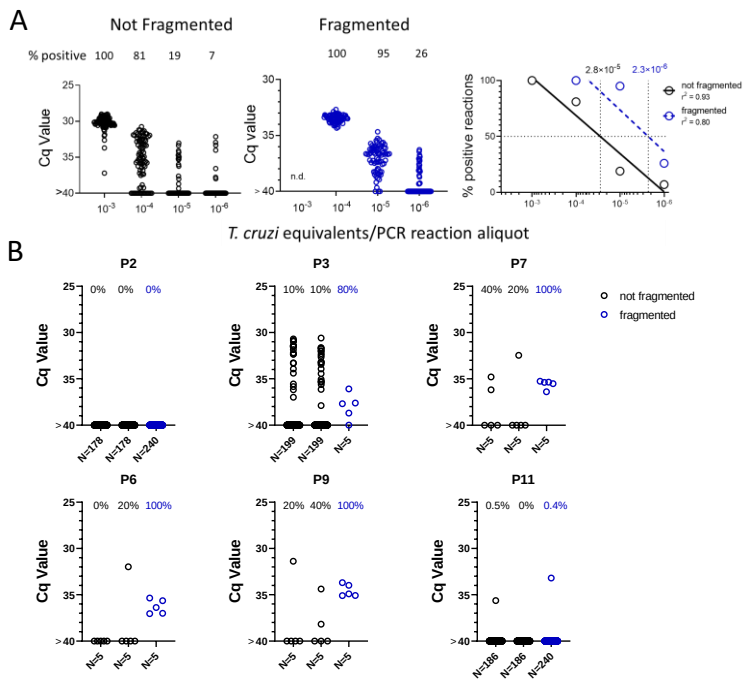

Figure 4. DNA Fragmentation increases the sensitivity of PCR detection of *T. cruzi* DNA. A) The quantity of parasite DNA required to yield 50% positive PCR reactions in replicate assays is reduced by >10-fold by cuphorn fragmentation of DNA from blood. A total of 72 to 96 replicate PCR reactions were 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

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

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

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

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

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

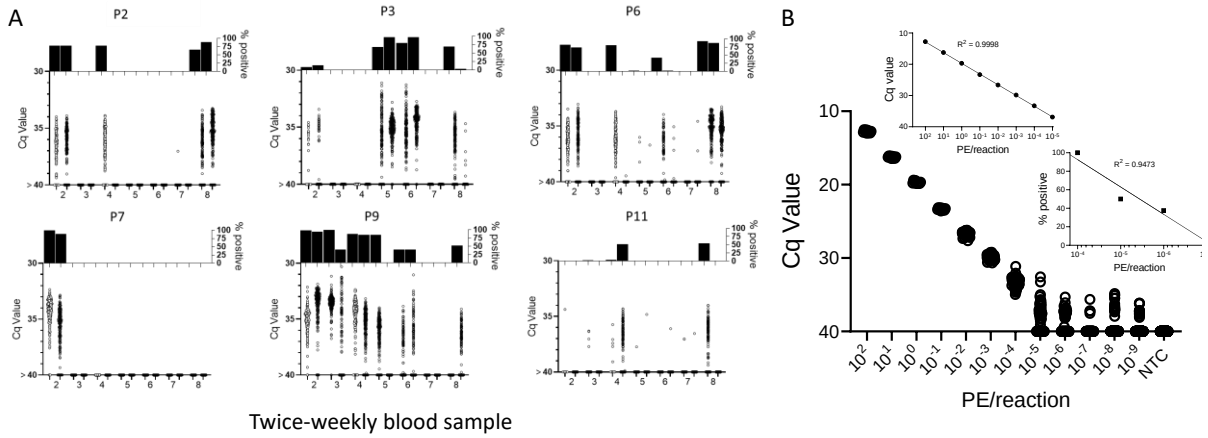

Figure 5. Repeat blood sample collection and deep-sampling PCR of fragmented DNA can quantify infection load over a minimum of 8 orders of magnitude. A) Frequent blood sampling demonstrates the sampling error involved in the detection of *T. cruzi* DNA in infected macaques with low parasite burden. Duplicate 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

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

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

270 <u>The blood cell pellet routinely contains the highest amount of detectable parasite DNA relative to other</u>
271 <u>blood fractions</u>

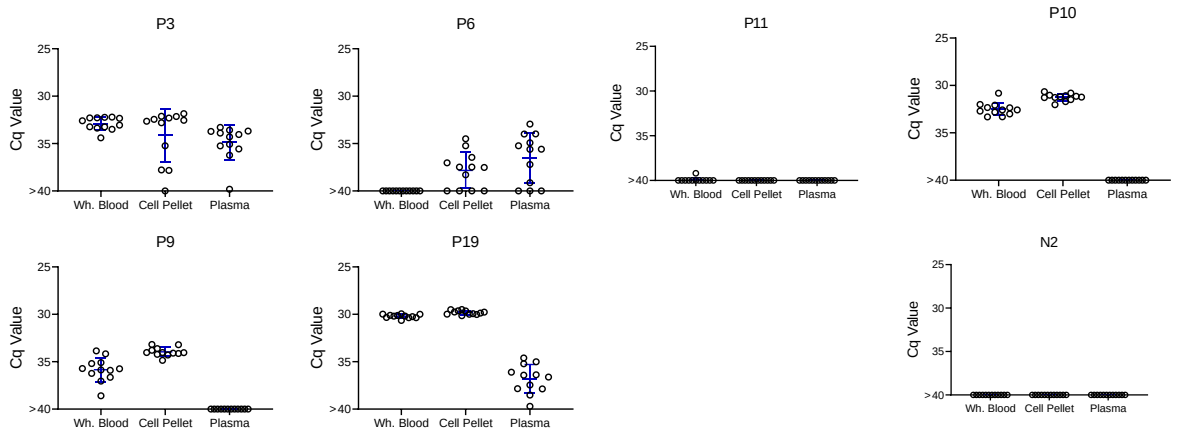

Figure 6. *T. cruzi* DNA can be detected in whole blood, the cell pellet of heparinzed blood, or plasma, but is at the highest and most consistently detected in the blood cell pellet. Replicate blood samples taken at the same time were either processed as whole blood (sample 1) or separated into the 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.

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

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

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

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

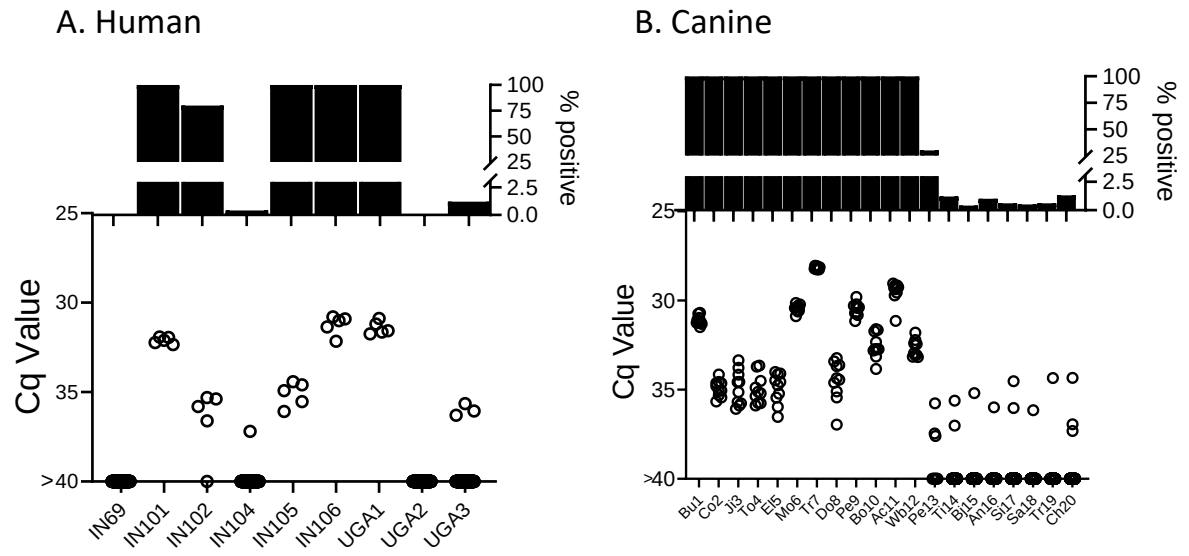

Figure 7. Deep sampling PCR of A) fragmented DNA from whole blood from 9 not-treated, chronically infected humans and B) fragmented blood cell pellet DNA from 20 seropositive dogs. An additional 10 seropositive dogs from this study group were negative for up to 384 replicate PCR reactions (not shown).

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.

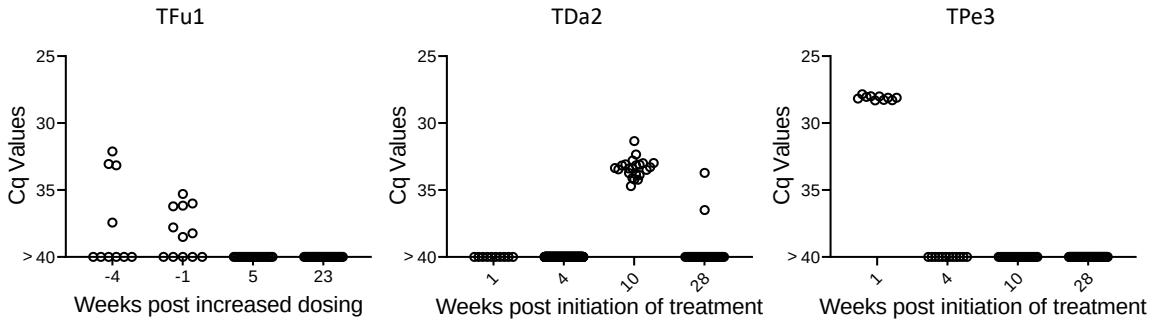

Figure 8. Monitoring by deep sampling PCR of *T. cruzi* DNA from whole blood in dogs during treatment with benznidazole. Dogs were treated with 18-20 mg/kg benznidazole twice (initially) or thrice (if remaining PCR positive) per week.

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

305 **Discussion**

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

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

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

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

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

368 In addition to providing much greater sensitivity for detecting active infection relative to conventional
369 single or low replicate (2-3/sample) PCR analysis, conducting 100's of replicate PCR reactions on
370 fragmented blood DNA also provides information on the relative abundance of parasites in the blood of
371 infected hosts. Most implementations of PCR for *T. cruzi* have a limit of consistent detection of ~10^{-3}
372 PE/assay, which equates to ~0.5 parasites/ml of blood in our assays. Combining sample DNA
373 fragmentation and deep-sampling extends the range of quantification to at least 10^{-6} PE per aliquot, or
374 ~0.00025 parasites/ml (1 parasite per 4 liters) of blood.

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

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

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

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

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

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

433 **Materials and Methods**

434 Macaques

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

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

452 Canines

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

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

466 Human

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

478 Blood DNA extraction and qPCR

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

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

498 Preventing contamination

499 To prevent contamination when preparing high numbers of qPCR reactions, a number of safeguards
500 were employed. The setup for all reactions was performed in a space separate from our main research
501 laboratory and in a class II A2 laminar flow hood otherwise not used for work with *T. cruzi* and using
502 supplies and instruments that were specific for this lab space. All components are sprayed with 70%
503 ethanol and DNA decontamination spray (LookOUT, Sigma) before being brought into the hood and all
504 hood surfaces are sprayed with ethanol and decontaminant at the end of each work session, followed
505 by UV exposure. PCR assay components were aliquoted from large batches into individual tubes
506 sufficient for each 384-well plate. The centrifuge used for DNA extractions was bleached and
507 decontaminated before processing the final elution for each DNA batch. DNA samples are diluted into
508 96 well plates and then loaded 96 wells at a time into the 384 well plate using the mini 96 pipette

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

515 DNA fragmentation

516 Purified DNA from blood was fragmented by either in a Covaris E220 Focused-ultrasonicator (which
517 allowed precise selection of target DNA fragment sizes of 1000, 500 or 300 bases), or using the 3in
518 cuphorn attachment to a Branson SFX250 Sonifier cooled by a Bio Rad 1000 mini chiller circulator. For
519 cuphorn sonication, DNA samples (typically 500 µl in volume and 15 µg to 150 µg total DNA) in 1.5 ml
520 Eppendorf snap cap tubes were wrapped securely in parafilm and kept on ice before fragmentation.
521 Sonifier settings were set to continuous mode with a time interval of 50 seconds and 60% amplitude for
522 5 cycles.

523 PCR standards

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

528 Hemoculture

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

532 Tissue PCR

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

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542

543 **Supplementary Information Captions**

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

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

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

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

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

554

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