



Published in final edited form as:

*Am J Trop Med Hyg.* 2009 August ; 81(2): 228–234.

## Polymerase Chain Reaction Detection of *Trypanosoma cruzi* in *Macaca fascicularis* Using Archived Tissues

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### Abstract

This study describes conventional and real-time polymerase chain reaction (PCR) methods developed to detect and quantify *Trypanosoma cruzi* DNA in cynomolgus monkeys (*Macaca fascicularis*) using formalin-fixed paraffin-embedded blocks archived for periods of up to 6 years. The highest concentration of *T. cruzi* DNA was found in the myocardium, urinary bladder, stomach, lymph node, adrenal gland, and colon. The concentration of *T. cruzi* DNA detected in cardiac tissues was 10–100-fold greater than found elsewhere; the mean concentrations of *T. cruzi* DNA in non-cardiac tissues were otherwise comparable. *Trypanosoma cruzi* DNA was amplified from cerebrum but not cerebellum or kidney. Successful use of DNA from formalin-fixed, paraffin-embedded blocks is important because most pathology laboratories routinely archive wax blocks. This archived resource can be used for further studies on the prevalence of this disease.

### INTRODUCTION

Chagas disease is an illness caused by infection with the kinetoplastid protozoan parasite *Trypanosoma cruzi*.<sup>1</sup> The parasite is typically transmitted to humans by blood-sucking insects belonging to the genus *Triatoma* (Order Hemiptera, Family Reduviidae) and commonly known as “cone-nosed” bugs. The disease is widespread in Central and South America, where currently 16–18 million people are estimated to be infected and 120 million are at risk of infection.<sup>2–4</sup> The parasite and reduviid insect vector are also present in the southern United States.<sup>5–10</sup> Away from rural areas and the natural environments that offer favorable habitats for the insect vector, infection is most commonly transmitted by blood transfusion and solid organ transplantation.<sup>11–14</sup> Chagas disease is also increasingly appreciated as a complicating opportunistic infection in immunocompromised individuals.<sup>15–19</sup> The numbers of infected individuals and individuals at risk of infection put Chagas disease in the same category as schistosomiasis and malaria as tropical health concerns of global significance.<sup>20</sup>

Southwest Foundation for Biomedical Research (SFBR) in San Antonio, Texas, is home to several thousand nonhuman primates, including baboons, chimpanzees, and various species of macaque, most of which are housed in large, open, outdoor cages. In 1984, it was discovered

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that some primates at SFBR were infected with *T. cruzi*.<sup>21</sup> Although the animals may be bitten by the insect vector while resting or sleeping, the primary route of infection is believed to be opportunistic consumption of the reduviid insect vector.<sup>22–26</sup> Commensal consumption of infected lice or other blood-sucking ectoparasites may also pose a significant exposure risk in nonhuman primates.<sup>27</sup>

At SFBR, conventional enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and immunochromatographic serodiagnostic kits are used routinely to screen colony animals for antibodies to *T. cruzi*. For the purposes of colony management, a positive outcome in at least two of these assay methods is accepted as an indication that the animal is infected with *T. cruzi*. These assays, which are designed to react to the presence of host antibodies specific to the parasite, are inexpensive and have excellent sensitivity.<sup>28–30</sup> Their specificity is good, although some cross-reactivity to antibodies for parasites having similar surface antigens has been reported and can generate false-positive results.<sup>30,31</sup> Alternative diagnostic methods such as hemoculture and xenodiagnosis have better specificity but rather poor sensitivity; they are also expensive and time-consuming to implement and impractical for routine screening of a large primate colony.<sup>32,33</sup> Direct microscopic visualization of the parasite in the host tissues is highly specific but much too insensitive and labor-intensive for routine application. Consequently, an independent, inexpensive, non-immunologic and non-microscopic assay for *T. cruzi* is needed to facilitate comprehensive testing and reliable detection of infection. Ideally, the method would be applicable retrospectively to archived tissue samples and could also provide a quantitative indication of the amount of parasite in various host tissues.<sup>34</sup>

Within the last decade assays using polymerase-chain reaction (PCR) for *T. cruzi* detection have matured as practical and robust alternatives to immunologic diagnosis that promise both high specificity and high sensitivity.<sup>35–37</sup> A comparison of PCR and standard microscopy for parasite detection showed that PCR assay exhibited greater sensitivity during both the acute and chronic phases of the disease, could consistently detect infections during the chronic stage that were intermittently recognizable microscopically, and gave earlier detection of infection.<sup>38</sup> Conventional and real-time PCR have been used to amplify *T. cruzi* DNA from infected mouse and human tissues and to isolate *T. cruzi* DNA from formalin-fixed paraffin-embedded (FFPE) sections of raccoon myocardium; nonhuman primate (*Papio* spp., *Macaca fascicularis*) myocardial, esophageal, and placental tissues; and human myocardial, adrenal, and gonadal tissues.<sup>26,39–43</sup>

The PCR methods have been described previously for diagnosis and definition of Chagas disease in rhesus (*Macaca mulatta*) and pigtailed (*Macaca nemestrina*) macaques.<sup>35,44</sup> We previously reported quantitative PCR detection of *T. cruzi* DNA in archived nonhuman primate tissues.<sup>39</sup>

Here, we describe the further development of conventional and real-time PCR methods to detect and quantify *T. cruzi* DNA in *M. fascicularis* (also known as cynomolgus macaque, crab-eating macaque, and long-tailed macaque) using tissues collected at necropsy and archived for periods of up to 6 years. These methods were applied retrospectively to a variety of tissue types from infected and uninfected monkeys.

## MATERIALS AND METHODS

### Study animals

The animals used for this study were 15 *M. fascicularis* maintained at SFBR and that died between 2000 and 2006. Demographic features of the animals are given in Table 1. In most cases the animal died of natural causes. Ten animals were selected retrospectively based on

the microscopic presence of *T. cruzi* organisms; five animals lacking microscopic evidence of organisms were chosen as controls. All animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee. The SFBR is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

### Tissue specimens

Complete necropsies had been performed on all animals. Tissue samples were harvested from various organs, processed conventionally, fixed in 10% neutral-buffered formalin, embedded in paraffin, and archived under ambient environmental conditions for periods of 1 to 6 years. The tissue types available for study were constrained by the retrospective nature of the samples; the most frequently available tissues in order of decreasing availability were cardiac muscle, colon, liver, spleen, kidney, adrenal, and cerebral cortex. Additionally, specimens of cerebellum, thyroid, lung, stomach, pancreas, small intestine, ovary, bladder, uterus, placenta, epidermis, subcutaneous adipose tissue, skeletal muscle, and lymph node were available for some animals. Blood or serum for retrospective immunologic diagnosis of infection was not available.

### DNA extraction

Samples were obtained from FFPE tissue blocks using a 3-mm tissue punch (Acuderm Inc., Fort Lauderdale, FL) and de-paraffinized overnight in xylene. The DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications and quantified by spectrophotometer (NanoDrop Technologies, Wilmington, DE). The concentration of DNA extracted from tissue samples ranged from 40 to 1046 ng/ $\mu$ L.

### Conventional PCR

Standard techniques of conventional PCR were used to test for the presence of parasite and host DNA in FFPE tissue samples from all 15 animals. Primers and probes used are listed in Table 2. Reference DNA for *T. cruzi* (Tulahuen strain) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Initially, a 188-bp repetitive nuclear DNA sequence specific to *T. cruzi* was amplified using primers TCZ1 and TCZ2.<sup>41</sup> Attempts to amplify *T. cruzi* DNA using standard *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) were unsuccessful, but consistent and reliable results were achieved using Platinum *Taq* DNA polymerase under "touchdown" conditions.<sup>45</sup> Each PCR reaction contained 2- $\mu$ L sample DNA, 1 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and other components as specified by the manufacturer, to a 25- $\mu$ L final volume.

In separate reactions, a 199-bp sequence of the human  $\beta$ -globin gene was amplified from *M. fascicularis* using the primer set PC03 and PC04.<sup>46</sup> Samples were run on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) under touchdown conditions. Briefly, following an initial denaturation step at 94°C for 2 minutes, the annealing temperature was set to 65°C for two cycles, and thereafter decreased by one degree for every 2 cycles until the temperature reached 58°C. At those conditions the samples were run for an additional 20 cycles. The PCR product was loaded on 2% agarose gels and electrophoresis was performed. They were then stained with ethidium bromide, visualized under UV illumination, and photo-documented. All reactions were run with positive and negative controls.

### Quantitative PCR

To study parasite tissue tropisms, we developed a real-time PCR protocol to quantify the amount of *T. cruzi* DNA present in various host tissues. Because of the limited availability of

samples, quantitative PCR was performed on only 6 animals that were found to be positive by conventional PCR.

For normalization of the results, commercially available kits were not appropriate for this application. For example, the TaqMan 18S ribosomal gene kit (Applied Biosystems) could not be used because *T. cruzi* is a eukaryotic organism, and use of this kit would result in the amplification of genomic DNA from both *M. fascicularis* and *T. cruzi*. It was also not possible to use the TaqMan GAPDH kit (Applied Biosystems), because the GAPDH gene sequence used in that kit is specific to a human sequence and not to *M. fascicularis*.

We therefore chose to use the peptidylprolyl isomerase A (PPIA) gene (also known as cyclophilin A or CyP A) as a control for normalizing the tissue amounts in different PCR reactions. The DNA extracted from FFPE blocks was adjusted to a concentration of 30 ng/μL for all samples. The PCR reactions were set up using the TaqMan 2× universal buffer (Applied Biosystems), primers and probe (Table 3), and 3-μL sample DNA. The reaction mixture was adjusted to a final volume of 20 μL. Primers and probe for quantitation of *T. cruzi* DNA were based on *T. cruzi* clone C1bb06 satellite gene sequence (Genebank accession no. AY520044). The PPIA gene sequence (Genebank accession no. DQ315473) for *M. fascicularis* was amplified separately and used as a control gene to normalize the samples. Primers and probes were designed using Primer Express software (Applied Biosystems). Real-time, fluorescence-based PCR was conducted using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

For each sample and standard a threshold cycle number ( $C_T$ ) was recorded. Standard curves relating to the starting concentration of *T. cruzi* DNA were constructed using a 10-fold serial dilution of DNA from the Tulahuen strain with concentrations ranging from 1740 to 0.0174 ng/μL. Samples were normalized by dividing the for *T. cruzi* DNA by the corresponding  $C_T$  value for the PPIA gene and multiplying by the mean  $C_T$  for PPIA of all samples. The resulting adjusted value was referred to the standard curve to determine the concentration of *T. cruzi* DNA in the samples.

## RESULTS

### Clinical symptoms

The clinical presentation of Chagas disease in *M. fascicularis* and other nonhuman primates was non-specific, typically involving lethargy, dehydration, colitis, steatosis, fecal pasting, and a history of diarrhea. The pathologic findings, however, were specific and consistent with Chagas disease. Gross diagnosis typically included pulmonary edema, ascites, hydrothorax, hydropericardium, hepatomegaly, myocarditis, and cardiomegaly. The diseased heart often had a flaccid, rounded appearance. A typical presentation is shown in Figure 1A.

### Histopathology

On microscopic observation, infected animals generally exhibited focal to diffuse myocardial infiltrates, primarily lymphocytic with minimum admixture of other inflammatory cells (Figure 1B), and commonly associated with focal necrosis. Clusters of organisms morphologically consistent with *T. cruzi* amastigotes were seen in some tissue sections (Figure 1C).

### Diagnosis

Infection with *T. cruzi* was confirmed using light microscopy of histologic sections from FFPE tissue blocks. The presence of one or more nests of *T. cruzi* amastigotes within cardiac myofibers was considered pathognomonic.

## Conventional PCR

*Trypanosoma cruzi* DNA was amplified from at least one tissue type from all 10 animals with histologically confirmed infections. Typical PCR results for cardiac and spleen tissue are shown in Figure 2. In particular, *T. cruzi* DNA was amplified from cardiac muscle, colon, liver, spleen, adrenal tissue, lymph node, lung, bladder, uterus, skin, stomach, skeletal muscle, and small intestine. Despite several attempts, *T. cruzi* DNA did not amplify from any of the kidney samples. The detection rate was highest (100%) in cardiac tissue, followed by colon (43%), spleen (29%), and adrenal tissue (29%). All five of the control animals were PCR negative in all tissues tested. The PCR results for the two groups are significantly different ( $\chi^2 = 14.96$ ,  $P < 0.001$ ).

## Quantitative PCR

The results of quantitative PCR are summarized in Figure 3, in which the concentrations of *T. cruzi* DNA amplified from various tissues are plotted for six infected *M. fascicularis* evaluated. With the exceptions of kidney and cerebellum, *T. cruzi* DNA was amplified from at least one sample of the available tissue types. The highest concentration was detected in cardiac tissue, and in amounts typically 10–100-fold greater than found elsewhere; the mean concentrations of *T. cruzi* DNA in non-cardiac tissues were otherwise broadly comparable. Cardiac tissue yielded variable but amplifiable amounts of material from all six infected animals and is clearly the most reliable source of parasite DNA. Bladder, thyroid, and colon tissue, although less reliable, did show the least variation between animals in the concentration of parasite DNA.

## DISCUSSION

The PCR study of FFPE tissues in *M. fascicularis* corroborates several tendencies that have been reported for *T. cruzi* infection in other species. It is evident that *T. cruzi* has the ability to invade many kinds of tissue, although the parasite preferentially infects host cardiac, digestive, and skeletal muscle tissues, and some tissues seem consistently to show higher parasitemia.<sup>2,26,40,47</sup> During the course of infection the highest concentrations of parasite DNA (and presumably parasite density) are found in the myocardium, underscoring the importance of this tissue for confident diagnosis of Chagas disease.<sup>2,48,49</sup>

In our sample *T. cruzi* DNA was also amplified from stomach and colon, confirming a tropism for the gastrointestinal system that has been reported in other species.<sup>26,47</sup> Unfortunately, esophageal tissue was not available to us, although it is another of the hollow muscular organs for which *T. cruzi* shows characteristic affinity.<sup>26,39</sup> Moderate amounts of *T. cruzi* DNA were present in adrenal tissue, and small amounts were present in the cerebrum, but not cerebellum, of some of the monkeys. Although *T. cruzi* has been observed in the cerebrospinal fluid of an infected patient, the parasite ordinarily shows little affinity for tissues of the central nervous system.<sup>50,51</sup> *Trypanosoma cruzi* was not amplified from any of the available kidney specimens, but it is clearly possible for *T. cruzi* to invade this organ and remain pathogenic.<sup>13,33,52</sup>

The variety of tissues available for analysis was limited by the retrospective nature of this study. However, future studies will expand the spectrum of tissue types to include adipocytes, which have been implicated as potential reservoirs for *T. cruzi* involved in parasite recrudescence; and placenta, for its involvement and possibly protective role in vertical transmission of infection.<sup>39,53</sup>

The data from real-time PCR have important implications for genetic analysis to identify genes influencing Chagas disease. Although all infected animals would be scored identically on the basis of conventional, qualitative, immunologic assays, it is evident from Figure 3 that most tissue types examined showed significant variability in the actual concentration of parasite

DNA. This *quantitative* variation is vastly more informative for genetic analysis than nominal information from immunologic assays and is the *sine qua non* of current statistical genetic methods for gene discovery.<sup>54,55</sup>

The ability to amplify *T. cruzi* DNA from tissues harvested at necropsy, even after processing, fixation, and years of storage, greatly expands the research potential of this resource.<sup>34</sup> Archived tissues can now be used to resolve the infection status of cases for which immunologic tests are consistently equivocal. Tissues other than blood can be assayed to obtain retrospectively additional diagnostic time points in the disease history of specific animals. Actual quantitation of *T. cruzi* DNA using real-time PCR can be used to generate information related to the progression of the disease, and to correlate parasite density and distribution with the condition of the animal at the time of death.<sup>48,56,57</sup> We are aware of a limitation of our study, namely the absence of immunologic tests in the same animals; however, in our previous work with archived baboon samples we showed that the presence of parasites in the tissue and not seropositivity *per se* correlated positively with amount of DNA detected.<sup>39</sup>

Natural infection of nonhuman primates at SFBR with *T. cruzi* is not limited to *M. fascicularis*; it has also been documented in baboons (*Papio hamadryas* spp.) and in one chimpanzee (*Pan troglodytes*) maintained in outdoor facilities.<sup>21,39</sup> The prevalence of infection is low (1–3% in baboons), but the presence of this under-recognized and essentially enzootic infection in these colony-raised primates may have important consequences for their use in biomedical research. Investigators should consider whether infection of experimental subjects with *T. cruzi*, and the sequelae of Chagas disease, could introduce significant confounding effects into experimental protocols.

## Acknowledgments

We gratefully acknowledge the technical assistance of Mary Jo Aivaliotis, Debbie Christian, Allen Ford, Sam Galindo, Janice MacRossin, Michael Torres, and Jane VandeBerg in collecting, processing, and assaying samples; Marie Silva, Antonio Perez for histopathology support; and Marie Tehas for photographic support. Mary Sparks provided expert assistance with data management and animal records research. We are also indebted to Tim Anderson, Nico Gouin, and Susan Mahaney for many helpful discussions.

Financial support: This research was supported in part by National Institutes of Health grants R01 RR016347 (to JTW) and P51 RR013986, and in part from facilities constructed with support from Research Facilities Improvement Program grants C06 RR015456 and C06 RR014578 from the National Center for Research Resources, National Institutes of Health.

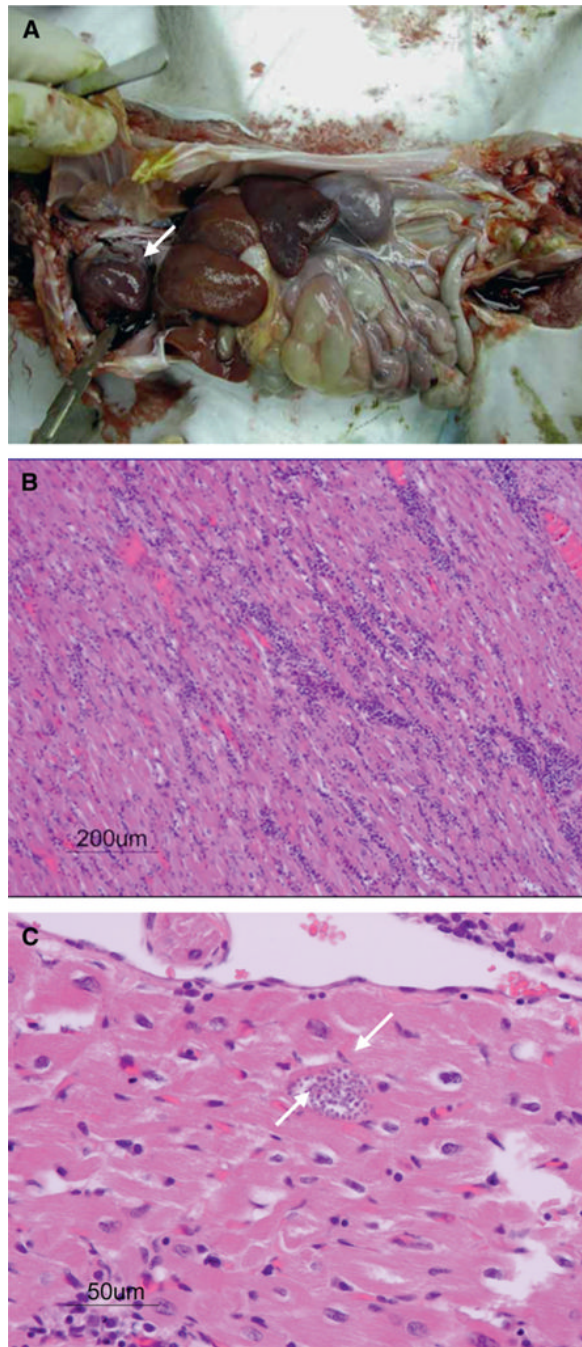
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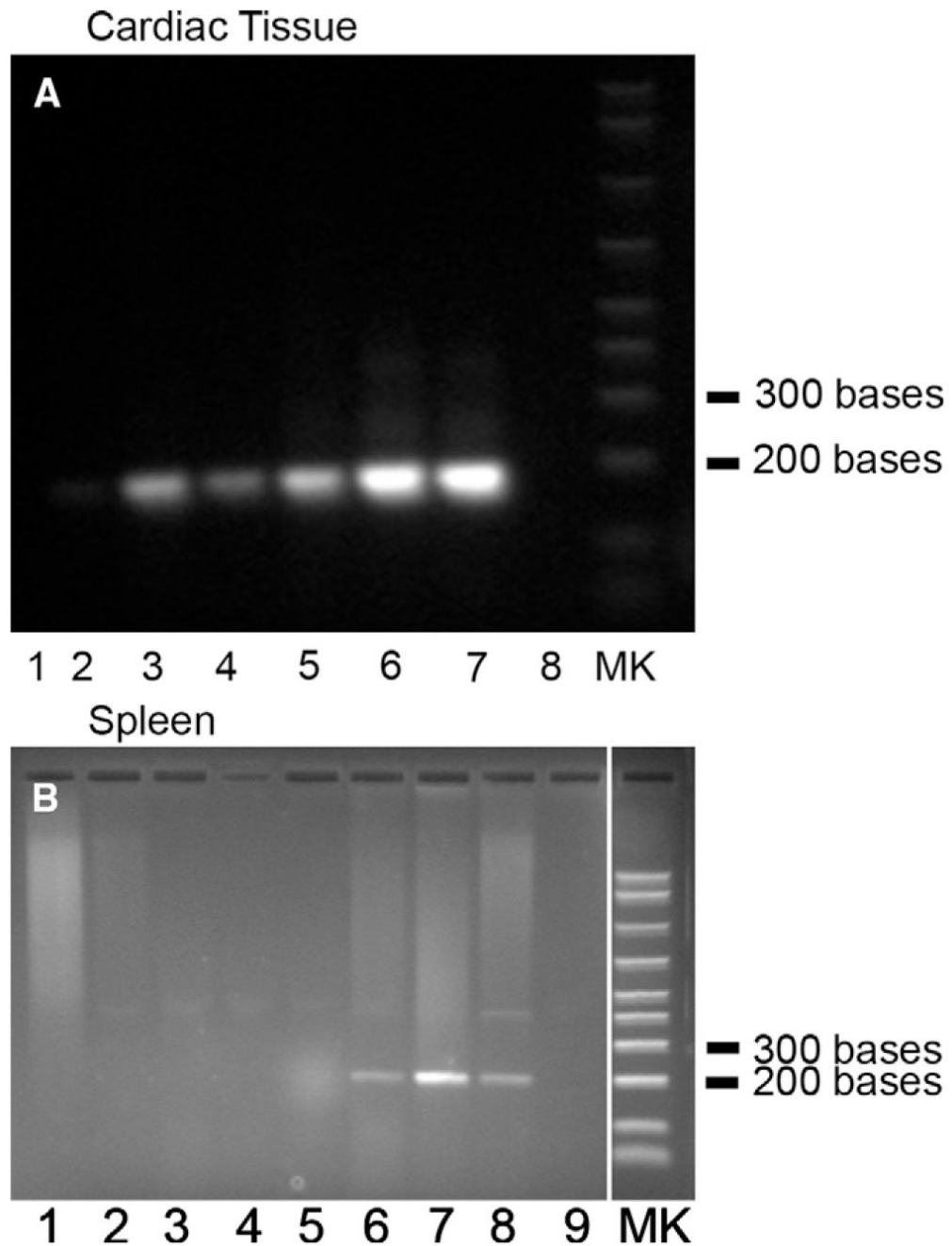
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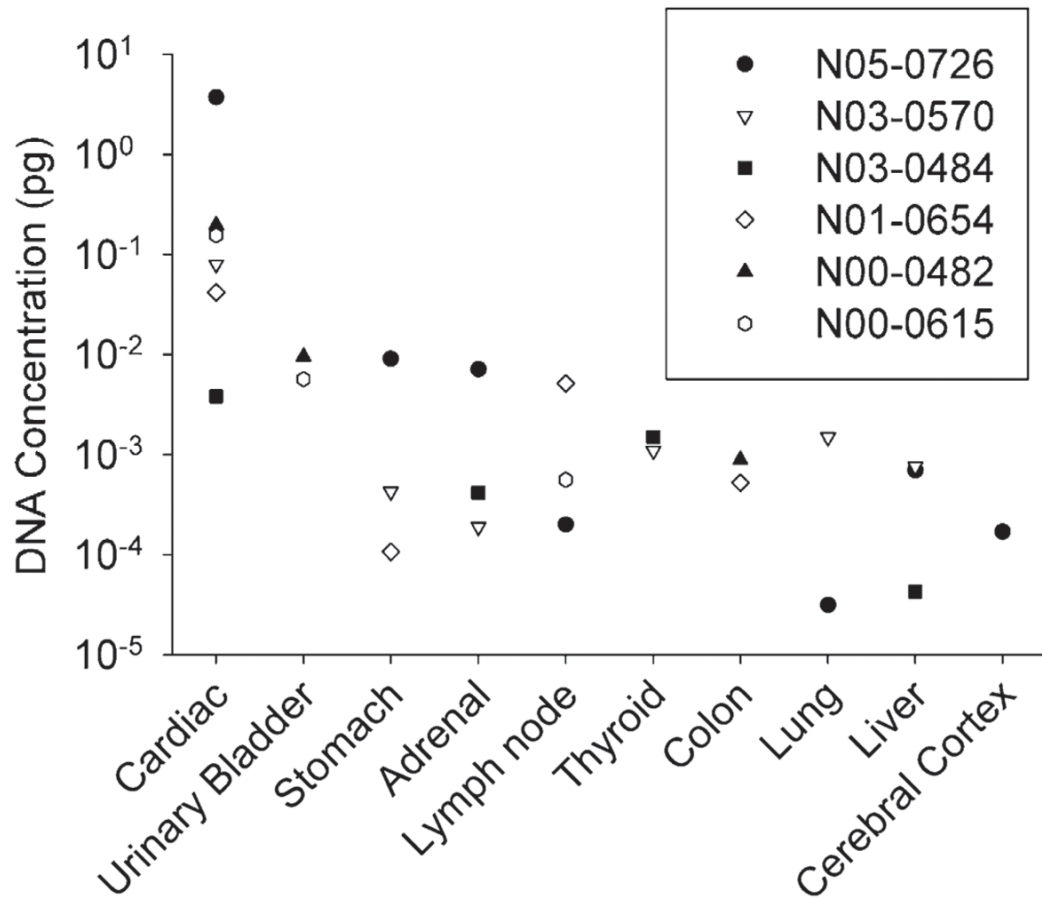
**Figure 1.**

**A**, Gross pathology in a 3-year-old *Macaca fascicularis* that died of naturally acquired Chagas disease, illustrating pulmonary edema, hydrothorax, hydropericardium, hepatomegaly, and rounded heart (arrow). Ascites have been drained. **B**, Histopathologic appearance of cardiac tissue in an infected *M. fascicularis*, illustrating the presence of focal to diffuse, primarily lymphocytic, myocardial infiltrates. H&E stain; bar = 200 µm. **C**, High power magnification of Panel **B** showing the presence within the cardiac muscle of a nest of organisms morphologically consistent with the amastigote form of *T. cruzi* (arrows). H&E stain; bar = 50 µm.



**Figure 2.**

**A**, Polymerase chain reaction (PCR) detection of *Trypanosoma cruzi* DNA in cardiac tissue using primers TCZ1 and TCZ2. Lane 1: control animal; Lanes 2–5: infected animals; Lanes 6–7: positive controls (*T. cruzi* DNA, Tulahuen strain); Lane 8: distilled water; lane MK: molecular weight markers. **B**, PCR detection of *T. cruzi* DNA in spleen using primers TCZ1 and TCZ2. Lanes 1–5: uninfected control animal; Lanes 6–8: infected animal; Lane 9: distilled water; lane MK: molecular weight markers.



**Figure 3.** Concentration of *Trypanosoma cruzi* DNA (in pg) by real-time polymerase chain reaction (PCR) in formalin-fixed paraffin-embedded (FFPE) tissue samples from six *Macaca fascicularis* positive for lymphocytic myocarditis and/or presence of *T. cruzi* organisms. Only non-zero concentrations are shown.

**Table 1**  
Demographic and pathologic features of the animals used in the study

Animal number	Demography		Pathology			PCR assay	
	Sex	Age (years)	Lymphocytic myocarditis	Case/control	<i>T. cruzi</i> organisms	Conventional	Real time
1	F	12.4	+	Case	+	Done	ND*
2	F	9.5	+	Case	+	Done	Done
3	F	1.58	+	Case	+	Done	Done
4	F	1.75	+	Case	+	Done	ND
5	F	1.92	+	Case	+	Done	Done
6	F	5.00	+	Case	+	Done	ND
7	M	1.67	+	Case	+	Done	Done
8	F	2.24	+	Case	+	Done	Done
9	F	0.5	+	Case	+	Done	Done
10	F	26	+	Case	-	Done	ND
11	F	4.92	-	Control	-	Done	ND
12	M	Stillborn	-	Control	-	Done	ND
13	M	Stillborn	-	Control	-	Done	ND
14	F	0.70	-	Control	-	Done	ND
15	M	Stillborn	-	Control	-	Done	ND

\* ND = Not done.

**Table 2**Primers used for detection of *Trypanosoma cruzi* using conventional polymerase chain reaction (PCR)

Target	Primer	Sequence	Reference
<i>T. cruzi</i> 188-bp sequence	TCZ1	5'-CGA GCT CTT GCC CAC ACG GGT GCT-3'	41
	TCZ2	5'-CCT CCA AGC AGC GGA TAG TTC AGG-3'	41
Human $\beta$ -globin	PC03	5'-ACA CAA ACT GTG TTC ACT AGC-3'	46
	PC04	5'-CAA CTT CAT CCA CGT TCA CC-3'	46

**Table 3**

Primers and probes used in real-time polymerase chain reaction (PCR) for quantification of *Trypanosoma cruzi* DNA

<i>T. cruzi</i>	Forward	5'-TGC ACT CGG CTG ATC GTT T-3'
	Reverse	5'-CAA AAA TTT AGA CCA CAA CGT GTG A-3'
	TaqMan probe	6FAM-CGA GCG GCT GCT G-MGBNFQ
Peptidylprolyl isomerase A (Cyclophilin A)	Forward	5'-GCA AAG TGA AAG AAG GCA TGA A-3'
	Reverse	5'-CCA TTC CTG GAC CCA AAG C-3'
	TaqMan probe	6FAM-TCC ATG GCC TCC ACA AT-MGBNFQ