Diagnosis of Chagas Disease by Detecting Species-Specific Repetitive Genomic DNA from Filtered Human Urine Samples

Miriam Price, BS; Nilanjan Lodh, PhD

ABSTRACT

Introduction: Chagas disease caused by *Trypanosoma cruzi* (*T cruzi*) found in the Americas is often missed during the early stage of infection due to lack of sensitive diagnostic tests. The classic immunological and parasitological tests often fail in the acute phase due to the nonspecific and low antibody level nature of the infection and in the chronic phase due to low levels of trypanosomes in the blood. For successful control strategies, there must be a sensitive and specific diagnostic test.

Objective/Methods: We have demonstrated the possibility (proof of concept) of detecting *T cruzi*-specific repeat DNA via polymerase chain reaction (PCR) by (1) spiking 15 urine samples collected from volunteers free of prior infection with 3 different concentrations of *T cruzi* (3 strains), *Trypanosoma brucei*, and *Trypanosoma rhodesiense* (African strain) genomic DNA and (2) from filtered collected clinical samples from Argentina. Three sets of primers were used.

Results: Our approach detected repeat DNA specific for *T cruzi* strains from 1 clinical sample by 2 sets of primer and from spiked urine by all 3 sets of primer but not the African species. A serial dilution (spiking) also was performed on *T cruzi* strains to detect sensitivities of the assay. One set of primers constantly detected satellite DNA for all *T cruzi* strains from 70 pg/µl to 175 fg/µl.

Conclusions: We were able to demonstrate the feasibility of detecting *T cruzi*-specific DNA from filtered urine samples by sensitive and specific PCR assay. Besides the evident increased sensitivity and specificity of primers, our approach can be used to explore Chagas prevalence in endemic areas – especially in congenital Chagas newborn screening – and in the acute phase.

INTRODUCTION

Chagas disease, also known as American trypanosomiasis, is a blood parasitic disease caused by the parasite *Trypanosoma cruzi* (*Tcruzi*), which is transmitted through the feces of the blood-sucking kissing bug.[1,2] The parasite is estimated to infect at least 8 million people in the Americas, with millions more at risk of infection[3] and confined mainly in parts of Mexico and Central and South America.[1] The classic parasitological and immunological tests often fail in the acute phase due to lack of symptoms and in the chronic phase due to low levels of trypanosomes in the blood.[4] Traditional diagnostic techniques, including blood smears, xenodiagnosis, haemoculture, and serology lack sensitivity, tend to produce false negatives, and are cumbersome to operate.[5,6] If control strategies are to succeed, there must be a reliable, robust, rapid, and inexpensive diagnostic test that is sensitive and specific enough to work even on noninvasive samples, such as urine, which can be used in developing countries.

Better diagnostics have been developed for Chagas detection to mostly address these issues. For example, sensitivity can be achieved by polymerase chain reaction (PCR) assay. In addition, its reliability, reproducibility with the minimal sample, rapid detection, and option for quantification (via quantitative PCR and digital droplet PCR) can provide an interesting opportunity to look for newborn screening and acute phase cases when trypomastigotes are much higher in the bloodstream. The assay also can be applied for the quantification of *T cruzi* infection in insect vectors. Recently, PCR was used on blood samples from Chagas-infected individuals.[6-8]

The above-mentioned studies focused on highly repetitive nuclear (n) or kinetoplast (k) DNAs. We choose 195 bp nDNA called satellite DNA, which comprises 9% of the genome[7] and has the following advantages over kDNA: (1) 1.8 times more copies of 195bp repeats of DNA, (2) the conserved nature of kDNA of

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Author Affiliations: Marquette University College of Health Sciences, Milwaukee, Wisconsin (Price, Lodh).

Corresponding Author: Nilanjan Lodh, PhD, Department of Medical Laboratory Science, 560 N 16th St, Schroeder Complex 274, Milwaukee, WI 53201; phone 414.288.3404; email nilanjan.lodh@marquette.edu; ORCID ID 0000-0002-0363-3538

T cruzi with other trypanosomatids, (3) nDNA of *T cruzi* differs from repeats of African trypanosomes, and (4) the high abundance and presence of nDNA in all *T cruzi* lineages (TcI-TcVI). The basis of this concept is that many nonreplicative trypomastigotes circulate in the bloodstream for 4 to 6 weeks during the acute phase of the infection.[2,6] Theoretically, parasite DNA can circulate through the blood filter through the kidney and end up in urine. It should be noted that the presence of species-specific DNA in the urine indicates that a viable infection is present.[9,10] We have demonstrated the presence of helminth and protozoan parasite species-specific DNA in urine in the past, such as *Schistosoma mansoni*,[11] *Schistosoma haematobium*,[12] *Strongyloides stercoralis*,[13] and *Plasmodium falciparum*.[14]

The current study detected *T cruzi*-specific nDNA from spiked filtered human urine samples and a small set of clinical samples. Our objective was to demonstrate the possibility of detecting *T cruzi*-specific satellite DNA fragments for 3 different strains via PCR from spiked human urine sediment captured on filter papers after filtration. We applied our approach to a small set of field-collected clinical samples from Argentina. We also intended to determine the sensitivity of 3 different primer sets through serial dilution and specificity by using *Trypanosoma brucei brucei* (*T b brucei*) and *Trypanosoma brucei rhodesiense* (*T b rhodesiense*) (African strains) genomic DNA as they do not share the same endemic area. Moreover, African trypanosomes will be useful in the future to distinguish co-endemic pathogens.

METHODS

Spiking of Urine Samples

Five different strains of Trypanosoma spp genomic DNA (BEI Resources, Manassas, Virginia) were used. Three strains were from 3 different geographic locations in South America: *T cruzi* CL (lineage: TcVI, NR-50237), *T cruzi* Dm28c (lineage: TcI, NR-50236), and *T cruzi* G (lineage: TcI, NR-50238). The other 2 strains were from Africa: *T b brucei* STIB 247 (NR-49829) and *T b rhodesiense* KETRI 243 (NR-49828). All 5 *Trypanosoma* spp genomic DNA were diluted (different concentrations) to imitate 3 different disease prevalence states – high (11ng/µl), medium (6 ng/µl), and low (1ng/µl) – resulting in a total of 15 different combinations (5 *Trypanosoma* spp and 3 concentrations for each).

Urine samples were collected from 3 volunteers from Marquette University who had never been exposed to Chagas disease. The pooled urine sample from the volunteers was divided into 15 combinations of 30 mL each and spiked with genomic DNA. The urine samples then were filtered through Whatman #3 filter paper (Pittsburg, Pennsylvania) and left to air dry on a clean bench surface with a net covering to prevent any contamination. The filter papers were packaged individually in Ziploc bags with a desiccant after drying to prevent degradation of the genetic material.

Table 1. Primer Sets Were Used for Species-Specific Repeat Satellite DNA Amplification of Different Strains for *Trypanosoma cruzi* by Polymerase Chain Reaction

Oligonucleotide Name	Oligonucleotide Sequence	Amplicon Size
TcA-F	5' ACA CGT TGT GGT CCA GAT TT 3'	118bp
TcA-R	5' GCG GAT AGT TCA GGG TTG TT 3'	
TcB-F	5′ CAG TCG GCG GAT CGT TT 3′	109bp
TcB-R	5′ AAC ACG TAG TGA CAG AGT GTG 3′	
TcC-F	5' CGG CTG CTG CAT CAC A 3'	112bp
TcC-R	5' TTG GTG TCC AGT GTG TGA AC 3'	

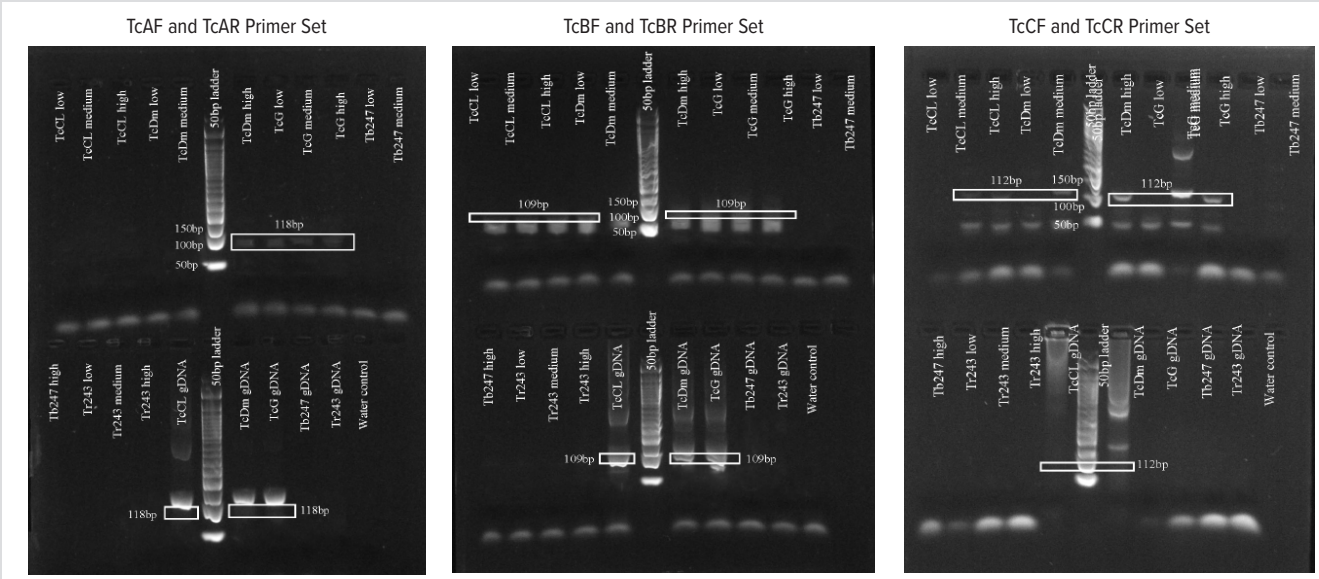
Table 2. Sensitivity of Detection of Diagnostic Target Repeat DNA in Serially Diluted Spiked Urine Samples by Three Sets of Primers

Trypanosoma spp	Primer Sets		
DNA Source	**TcAF & TcAR (118 bp)**	**TcBF & TcBR (109 bp)**	**TcCF & TcCR (112 bp)**
***Trypanosoma cruzi* CL**			
Low I (70 pg/µl)	Negative	Positive	Positive
Low II (4 pg/µl)	Positive	Positive	Negative
Low III (175 fg/µl)	Positive	Positive	Positive
Med I (70 pg/µl)	Negative	Negative	Negative
Med II (4 pg/µl)	Positive	Positive	Positive
Med III (175 fg/µl)	Positive	Positive	Negative
High I (70 pg/µl)	Negative	Negative	Positive
High II (4 pg/µl)	Positive	Negative	Negative
High III (175 fg/µl)	Positive	Positive	Positive
***Trypanosoma cruzi* Dm28c**			
Low I (70 pg/µl)	Negative	Positive	Positive
Low II (4 pg/µl)	Negative	Positive	Positive
Low III (175 fg/µl)	Positive	Positive	Positive
Med I (70 pg/µl)	Positive	Positive	Positive
Med II (4 pg/µl)	Positive	Positive	Positive
Med III (175 fg/µl)	Positive	Positive	Negative
High I (70 pg/µl)	Positive	Positive	Positive
High II (4 pg/µl)	Positive	Positive	Positive
High III (175 fg/µl)	Negative	Positive	Negative
***Trypanosoma cruzi* G**			
Low I (70 pg/µl)	Negative	Positive	Positive
Low II (4 pg/µl)	Positive	Positive	Negative
Low III (175 fg/µl)	Positive	Positive	Negative
Med I (70 pg/µl)	Positive	Positive	Positive
Med II (4 pg/µl)	Positive	Positive	Positive
Med III (175 fg/µl)	Positive	Positive	Negative
High I (70 pg/µl)	Positive	Positive	Positive
High II (4 pg/µl)	Positive	Positive	Negative
High III (175 fg/µl)	Positive	Positive	Negative

DNA Extraction From Spiked Urine Samples

DNA extraction was performed for all filter papers. A total of 12 discs (~1mm diameter) were punched out from a single quadrant of a filter paper with a regular paper punch (4 quadrants were made by folding the filter paper twice). All discs were used to extract DNA by QIAmp DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) by modifying the manufacturer's protocol.[15] Before following the manufacturer's protocol, all the paper discs

Figure. Agarose Gel Images of the Amplified Repeat Fragment From Spiked Urine Samples for *Trypanosoma cruzi* Strains With Three Different Sets of Species-Specific Primers[a]



Abbreviations: TcCL, *T cruzi* strain CL; TcDm, *T cruzi* strain Dm28c; TcG, *T cruzi* strain G; Tb247, *T brucei* strain STIB 247; Tr243, *T brucei* strain KETRI 243*; gDNA, genomic DNA.*

[a]The predicted amplicons have been shown boxed.

from each sample were placed into a 1.5 mL Eppendorf tube with 600 µl nuclease-free water. The tubes were incubated at 95 °C for 10 minutes and then put on a rotator at room temperature overnight (12–14 hours). The next morning, the water-containing DNA was transferred to a Qiagen QIAmp 2 mL column tube. Then the DNA was precipitated and concentrated using the QIAmpDNA Blood Mini Kit (Qiagen, Maryland) according to the manufacturer's protocol.[11] Then concentrations of extracted DNA were measured by NanoDrop (Thermo Scientific, Wilmington, Delaware), and 2 aliquots were stored at -20 °C for PCR amplification.

Primers and Serial Dilution of Spiked Samples

Three sets of primers–TcA, TcB, and TcC (Table 1)–were designed for PCR amplification based on 195bp satellite DNA sequence using the PrimerQuest online tool (Integrated DNA Technologies, Coralville, Iowa). A serial dilution was performed on 9 spiked urine samples (for each low, medium, and high concentration) for 3 *T cruzi* strains (CL, Dm28c, and G). For each combination, such as *T cruzi* CL low, 3 dilutions were made: low I (70 pg/µl), then 20-fold dilution to low II (4pg/µl), and another 20-fold dilution to low III (175 fg/µl). This was repeated for the other 8 combinations (Table 2), and all dilutions were named accordingly based on their initial concentration. In this way, a total of 27 different combinations was obtained (Table 2). The concentration of all the serially diluted spiked urine samples was measured via NanoDrop and was stored at -20 °C for PCR amplification. All 27 dilutions were tested with 3 sets of the above-mentioned primers to determine the sensitivity and specificity of each pair of primers.

PCR Amplification of Spiked Samples

For every reaction, 4 different controls were used: (1) *T cruzi* genomic DNA as positive control, (2) African trypanosome genomic DNA as negative control, (3) extracted DNA from urine collected in-house as negative control, and (4) nuclease-free water as no DNA control (Sigma-Aldrich, St. Louis, Missouri). PCR amplification was in a 10 µL volume with Taq 2X Mastermix (New England Biolabs, Ipswich, Massachusetts), 0.5 µL of 10 µM of each primer, 1 to 2 µL (5-15 ng/µL) of DNA, and PCR-grade water (Sigma-Aldrich, St. Louis, Missouri). Three sets of primer were used to amplify repeat nDNA specific to *T cruzi* as follows: initial denaturation at 95 °C for 10 minutes was followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 90 seconds and extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. Amplified PCR products were visualized by 2% agarose gel stained with SYBR Green (Thermo Scientific, Waltham, Massachusetts) with a 50 bp reference ladder (New England Biolabs, Ipswich, Mass) to compare amplified fragment size. All agarose gels were visualized in the Azure C200 gel documentation system (Azure Biosystems, Dublin, California).

PCR Amplification of Clinical Samples

Five field-collected filtered urine samples collected in Argentina were received from Johns Hopkins University. DNA was extracted

by the above-mentioned approach through QIAmp DNeasy Blood and Tissue Kit amplified by PCR (DNA concentration: 1ng/µl) and visualized by gel electrophoresis. The identity of the amplicons was confirmed by sequencing (GENEWIZ, Plainfield, New Jersey) of the PCR product and matched against the GenBank sequence (BLAST, National Center for Biotechnology Information [NCBI], Bethesda, Maryland).

Table 3. Detection by Three Sets of Primers of Diagnostic Target Repeat Satellite DNA Spiked in Human Urine

Trypanosoma spp		Primer sets[a]		
DNA source	**Infection Prevalence**	**TcAF & TcAR (118 bp)**	**TcBF & TcBR (109 bp)**	**TcCF & TcCR (112 bp)**
***Trypanosoma cruzi* CL**				
Genomic DNA	Low	Negative	Positive	Negative
	Medium	Negative	Negative	Positive
	High	Positive	Positive	Positive
***Trypanosoma cruzi* Dm28c**				
Genomic DNA	Low	Negative	Negative	Negative
	Medium	Negative	Negative	Negative
	High	Positive	Positive	Positive
***Trypanosoma cruzi* G**				
Genomic DNA	Low	Negative	Positive	Negative
	Medium	Positive	Positive	Positive
	High	Positive	Positive	Positive
***Trypanosoma brucei* 247**				
Genomic DNA	Low	Negative	Negative	Negative
	Medium	Negative	Negative	Negative
	High	Negative	Negative	Negative
***Trypanosoma rhodesiense* 243**				
Genomic DNA	Low	Negative	Negative	Negative
	Medium	Negative	Negative	Negative
	High	Negative	Negative	Negative

[a]There are 5 independent amplifications for each primer set for each combination. The cumulative result includes all positive or all negative results by each independent amplification.

RESULTS

Detection in Spiked Urine Samples

For 3 different types of serially diluted spiked urine samples imitating low, medium, and high infection prevalence, species-specific repeat nDNA was detected from all 3 concentrations (70 pg/µl to 175 fg/µl) for each type, indicating the detection limit of the designed PCR assay, which is less than 1 copy of the target nDNA fragment. All 3 different geographical *T cruzi* strains were detected by 3 different sets of designed primers. In addition, the TcB primer set detected all 3 serial dilutions for each type for both *T cruzi* Dm28c and G strains. It was not detected on 3 occasions (medium I: 70 pg/µl, high I and II: 70 pg/µl, and 4 pg/µl) for *T cruzi* CL strain. The detection was more consistent compared to the other 2 primer sets (Table 2).

We found that species-specific repeat nDNA was detected from spiked urine samples for all 3 *T cruzi* strains (Figure) for multiple runs (5 runs per sample) of PCR. nDNA was not detected from *Tb brucei* and *Tb rhodesiense* (African strains) spiked urine samples, indicating the conserved nature of the repeat fragment within the satellite DNA of the *T cruzi*. The designed primers specifically detected only *T cruzi* repeat nDNA without any cross-amplification. TcB primers detected *T cruzi* repeat nDNA for all African strains for all 3 genomic DNA serial dilutions compared to 2 other sets of primer (Figure, Table 3).

Detection in Clinical Urine Samples

One clinical sample was positive for *T cruzi* by TcA-F/ TcA-R and TcC-F/TcC-R sets of primers (Figure 2). There was no amplification from the African strains genomic DNA controls and water control. The sequenced amplicons amplified by 2 sets of primers were matched against the NCBI GenBank for the identity of the amplified fragment. For TcA-F/ TcA-R, a 195bp satellite DNA (GenBank: AY520029) came out as the top match with 98% query coverage and 86% identity. For TcC-F/TcC-R, the amplicon matched with another 195bp satellite DNA (GenBank: FJ768490) with 100% query coverage and 97% identity. The identity matching determined the amplification of the targeted *T cruzi*-specific repeat nDNA of the designed species-specific primers and the uniqueness of the repeat fragment belonging to *T cruzi*.

DISCUSSION

We were able to demonstrate the feasibility of identifying *T cruzi*-specific satellite DNA from spiked filtered human urine samples and field-collected samples by sensitive and specific PCR assay. The positive detection was achieved for all 3 South American strains but not for African trypanosome species. This demonstrates the specificity of our assay. All 3 primer sets consistently detected only *T cruzi* satellite DNA; in particular, primer set TcBF/ TcBR was most sensitive in detecting different levels of DNA from different *T cruzi* strains (Table 2). The detection sensitivity of our assay (to 175fg/µl, Table 3) should facilitate the examination of asymptomatic infection, especially in the acute phase when effective intervention is most likely. Again, the TcB-F/ TcB-R primer set (Table 2) provided us with promising results to try this assay for field-collected samples in the future.

The detection of *T cruzi*-specific repeat DNA fragments from 1 clinical sample is evidence for the possibility of transmission of parasite DNA via the bloodstream into the urine. The presence

of parasite DNA in the amplified PCR product has been confirmed by sequencing and matching against the *T cruzi* satellite DNA, which is our target biomarker for this assay. This presence might be an indication of active infection or DNA coming from dead parasites. This needs to be determined by implementing drug intervention studies and detecting parasite DNA before and after intervention from the same individuals. Recently, *T cruzi*-specific antigens have been detected in urine by the Chagas Urine Nanoparticle Test (Chunap) as part of early and noninvasive diagnosis of congenital Chagas diseases.[16,17]

However, we acknowledge the limitations of the lab-based nature of our study, that spiking genomic DNA in human urine may not exactly replicate/imitate the nature of the disease in endemic countries. We evaluated a very small set of field-collected samples, and the timing of the shedding of DNA in urine is not determined in the study. A bigger sample size collected from different prevalence areas of endemic countries, from patients with different infection status, from infection with different *T cruzi* lineages, and comparing urine samples against blood samples will provide a better estimate of the assay and comparing urine-based PCR assay against parasitological and immunological assay. Additionally, exploring other repeat biomarkers specific to *T cruzi* with next-generation sequencing and bioinformatics approach will improve the application of the assay in endemic countries. Also, the time point of shedding of DNA in urine will be determined once the sensitivity and specificity of the performed test in a larger clinical setting is determined.

CONCLUSIONS

We sought to detect *T cruzi*-specific DNA via PCR from filtered urine samples collected from individuals living in the endemic countries to determine the presence of infection or absence of Chagas disease and to compare our assay against other traditional diagnostics assays for both acute and chronic disease phase. The accuracy of the designed primers and specificity of our PCR assay demonstrated the feasibility of identifying the Chagas disease causing parasite-specific repeat nDNA from filtered urine from both spiked and clinical samples by sensitive and specific PCR assay.

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