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TECHNICAL ADVANCE

Toward the Establishment of a Single Standard Curve for Quantification of *Trypanosoma cruzi* Natural Populations Using a Synthetic Satellite Unit DNA Sequence

Arturo Muñoz-Calderón,* Natalia Lins Silva-Gomess,† Sofia Apodaca,* Belkisyolé Alarcón de Noya,‡ Zoraida Díaz-Bello,‡ Leticia Rocha Quintino Souza,† Alexandre Dias Tavares Costa,§ Constança Britto,† Otacilio Cruz Moreira,† and Alejandro G. Schijman*

From the Laboratorio de Biología Molecular de la Enfermedad de Chagas, Instituto de Ingeniería Genética y Biología Molecular "Dr Héctor Torres" (INGEBI), Buenos Aires, Argentina; the Laboratório de Biologia Molecular e Doenças Endêmicas,† Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil; the Sección de Inmunología,‡ Instituto de Medicina Tropical "Dr Félix Pifano," Universidad Central de Venezuela, Caracas, Venezuela; and the Laboratório de Ciências e Tecnologias Aplicadas à Saúde (LaCTAS),§ Instituto Carlos Chagas (ICC), Fundação Oswaldo Cruz, Curitiba, Brazil

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Address correspondence to Alejandro G. Schijman, Ph.D., Laboratorio de Biología Molecular de la Enfermedad de Chagas, Instituto de Ingeniería Genética y Biología Molecular "Dr Héctor Torres" (INGEBI), Buenos Aires, Argentina; or Otacilio Cruz Moreira, Ph.D., Plataforma de PCR em Tempo Real RPT09A, Laboratório de Biologia Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. E-mail: otacilio@ioc.fiocruz.br or schijman@dna.uba.ar.

Accurate diagnostic tools and surrogate markers of parasitologic response to treatment are needed for managing Chagas disease. Quantitative real-time PCR (qPCR) is used for treatment monitoring, but variability in copy dosage and sequences of molecular target genes among different *Trypanosoma cruzi* strains limit the precision of quantitative measures. To improve qPCR quantification accuracy, we designed and evaluated a synthetic DNA molecule containing a satellite DNA (satDNA) repeat unit as standard for quantification of *T. cruzi* loads in clinical samples, independently of the parasite strain. Probit regression analysis established for Dm28c (TcI) and CL-Brener (TcVI) stocks similar 95% limit of detection values [0.903 (0.745 to 1.497) and 0.667 (CI, 0.113 to 3.927) copy numbers/μL, respectively] when synthetic DNA was the standard for quantification, allowing direct comparison of loads in samples infected with different discrete typing units. This standard curve was evaluated in 205 samples (38 acute oral and 19 chronic Chagas disease patients) from different geographical areas infected with various genotypes, including samples obtained during treatment follow-up; high agreement with parasitic load trends using standard curves based on DNA extracted from spiked blood with counted parasites was obtained. This qPCR-based quantification strategy will be a valuable tool in phase 3 clinical trials, to follow up patients under treatment or at risk of reactivation, and in experimental models using different parasite strains. *(J Mol Diagn 2021, ■: 1—12; https://doi.org/10.1016/j.jmoldx.2021.01.007)*

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, affects mostly vulnerable populations in 21 countries of the Americas, with an annual incidence of 30,000 cases and 9000 newborns becoming infected during gestation. CD affects approximately 6 million people and causes, on average, about 14,000 deaths per year.[1] In the last decade, this neglected tropical disease has become a global concern because of the increasing migration from Latin America to nonendemic countries.[2,3]

Accurate diagnostic tools and surrogate markers of parasitologic response to treatment are priorities in CD research and development.[4] To develop an accurate laboratory tool for diagnosis and treatment follow-up of patients

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with CD, several issues have to be addressed. These include the low and intermittent parasitic burden during the chronic phase of infection and the parasite genotype diversity, as *T. cruzi* discrete typing units (DTUs), TcI to TcVI and Tcbat, are unevenly distributed in different geographical regions.[5]

Quantitative real-time PCR (qPCR)-based assays have been proposed to fill in these gaps,[6–8] and analytical validations following international guidelines[9–11] and harmonization studies using artificial and clinical specimens have to be conducted before this laboratory tool can be confidently used in clinical trials and practice.[12–15] Thus far, standardized operative procedures based on the few available validated qPCR protocols have been applied mostly for detection of acute infections due to congenital transmission, oral outbreaks, and organ transplantation,[15–19] as well as to provide a surrogate marker of treatment failure in the context of clinical trials.[14,20–22]

In a few studies, qPCR analysis of clinical samples has been accompanied by the establishment of external quality assurance systems to enable monitoring the performance of the methodology through proficiency testing panels and retesting of clinical samples by the different laboratories involved.[20,23] However, difficulties were encountered regarding the trueness and precision of quantitative measurements; these difficulties occurred mostly in multicenter clinical trials that covered patients' cohorts from various countries where different *T. cruzi* genotypes prevail. An explanation for this heterogeneity among quantitative results is that the gene dosage of the molecular targets used for qPCR, especially the repetitive satellite DNA (satDNA) sequence,[24] varies among parasite strains.[7,12,14,24–26] This scenario has led to the need for quantitative standard curves built up with DNA isolated from parasite stocks representative of the DTUs prevailing in each clinical setting and geographical area.[13–15] However, this approach makes it difficult to perform robust meta-analyses of parasitic loads obtained in laboratories that test samples from patients infected with different *T. cruzi* genotypes. Moreover, it has been reported that patients may be infected with mixed parasite populations belonging to different DTUs.[5,16–18]

In this context, we designed and evaluated a synthetic oligonucleotide with the satDNA sequence repeat to be used as the DNA standard for quantification of *T. cruzi* loads independently of the parasite genotype infecting the patients under study.

Materials and Methods

Ethics Statement

The samples used in this work were derived from previous studies approved by the bioethical committees of the participating institutions, according to the principles expressed in the Declaration of Helsinki. Written informed consent forms were signed by the adult study subjects and by parents/guardians on behalf of all minor subjects. All samples were pre-existent at the time of this study and were anonymized before being processed.

All patients from Venezuela signed an informed consent form, approved by the Ethics Committee of the Institute of Tropical Medicine, Faculty of Medicine, Universidad Central de Venezuela (CEC-IMT 019/2010, December 10, 2010). All patients from Brazil also signed an informed consent form, approved by the Ethics Committee of the Fundação Oswaldo Cruz (CEP IPEC 007/2007).

DNA Extraction

Blood samples were obtained and immediately mixed with an equal volume of 6 mol/L guanidinium hydrochloride/0.2 mol/L EDTA, pH 8.00 [guanidine EDTA buffer (GEB)].[12] After 24 to 48 hours at room temperature, GEB samples were stored at 4°C for DNA extraction and qPCR analysis. Three hundred microliters of GEB samples were processed with the High Pure PCR Template Preparation kit (Roche Diagnostics Corp., Indianapolis, IN) as described in Duffy et al.[13]

To build the standard curves for quantification of parasitic loads, DNA from spiked blood samples was obtained in the same way as reported for the clinical samples. DNA eluates were stored at −20°C until use in qPCR analysis.

satDNA Oligonucleotide for Construction of a qPCR Quantification Standard Curve

The satDNA repeats from strains and clones representative of the DTUs I to VI available at the GenBank were aligned, showing the high conservation of the repeat (Supplemental Figure S1). A 166-bp region of *T. cruzi* nuclear satDNA was amplified from *T. cruzi* Y strain DNA (TcII) by conventional PCR, using Cruzi 1 and Cruzi 2 primers.[6] The PCR product was excised from an agarose gel and purified by using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL), according to the manufacturer's instructions. The PCR product was cloned by using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Three positive clones were isolated and submitted to DNA sequencing using the ABI 3730xl 96-capillary DNA analyzer (Applied Biosystems, Foster City, CA) on the DNA sequencing Platform RPT01A from the Oswaldo Cruz Foundation, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed by using Sequence Scanner v1.0 (Applied Biosystems) and aligned against a nuclear satDNA reference sequence (*https://www.ncbi.nlm.nih.gov/nuccore*, GenBank accession number AY520087.1; *T. cruzi* clone YA05 satellite sequence, from Y strain) with Mega Software v4.0.2 software. A sequence containing the majority single nucleotide polymorphisms among the cloned sequences was chosen to produce the synthetic oligonucleotide (gBlock Gene Fragments, Integrated DNA Technologies, Coralville, IA) (Table 1). Its concentration was estimated by using an ND 2000 spectrophotometer

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Table 1 Sequences of *Trypanosoma cruzi* satDNA and IAC Synthetic DNA Fragments

Target	Sequence	Length of fragment (bp)	Sequence ID NCBI	Description	Query cover	Identity	E value
Nuclear *Satellite* DNA	5′-AGTCGGCTGATCGTTTTCGAGCGGCTGC TACATCACACGTTGTGGTCTAAATTTTTG TTTCCAATTATGAATGGTGGGAGTCAGA GGCACTCTCTGTCACTATCTGTTTGCGTG TTCACACACTGGACACCAAACAACCCTG AATTATCCGCTGCTTGGAGGAATT-3′	166	XM_805,618.1	*T. cruzi* strain CL-Brener hypothetical protein Tc00.1047053508097.10 partial mRNA	98%	97%	3×10^{-74}
Exogenous internal positive control (IAC)	5′-ACCGTCATGGAACAGCACGTACCGATTTA TAAGATTGCTGGAGAAATGACTGGATTTG GAGCATCTGTTCTTGAAGGTGTTTTAGCTT TCGTCTTGGTTTATACTGTGTTCACGGCTA GCGATCCCAGACGTGGGCTACCTTTAGCA GTGGGACCTATATTTATAGGGTTTGT TGCGGGAG-3′	181	NM_114,612.3	*Arabidopsis thaliana* putative aquaporin TIP5-1 mRNA, complete cds	100%	100%	2×10^{-88}

Sequence identifiers (IDs) available from https://www.ncbi.nlm.nih.gov. IAC, internal amplification control; NCBI, National Center for Biotechnology Information; satDNA, satellite DNA. Q27

(NanoDrop, Thermo Fisher Scientific, Waltham, MA) and the copy number determined by using the DNA Copy Number Calculator (Thermo Fisher Scientific) considering the DNA fragment length and the molar mass per bp.

Duplex Real-Time qPCR Procedures

The standardized satDNA qPCR assay used the Cruzi 1 and Cruzi 2 primers and Cruzi 3 probe reported by Piron et al[6] and the exogenous internal amplification control reported by Duffy et al.[7] The reaction was performed with 5 μL of resuspended DNA using FastStart Universal Probe Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) in a final volume of 20 μL. Optimal cycling conditions for both qPCR targets were a first step of 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds, and 58°C for 1 minute. The amplifications were performed by using Rotor-Gene 6000 (Corbett Life Science, Cambridgeshire, UK) or ABI7500 (Applied Biosystems) devices.

Construction of Standard Curves for qPCR Quantification

To conduct comparative qPCR experiments using the novel synthetic satDNA sequence as standard and genomic DNA (gDNA) isolated from cultured parasites, two types of standard curves were constructed, as discussed in the following sections.

Standard Curve Using Blood Spiked with Cultured Parasites

Q11 This type of standard curve was plotted with total DNA obtained from a seronegative-GEB sample, spiked with 10^5 parasite equivalents/mL of blood; 1/10 serial dilutions were performed, using as a matrix total DNA obtained from a pool of blood samples from seronegative individuals, as reported in previous studies.[13] *T. cruzi* DTU I (Dm 28c clone) and DTU VI (CL-Brener clone) gDNA-based standard curves were constructed to quantify parasitic loads.

Standard Curve Using Synthetic satDNA

To build this standard curve, total DNA obtained from a GEB sample from a seronegative person was mixed with the synthetic *T. cruzi* satDNA oligonucleotide at 10^8 copies/μL, and 1/10 serial dilutions were performed by using DNA obtained from a pool of seronegative individuals as a matrix, ranging from 10^5 to 1 satDNA copies/μL.

Comparison of satDNA Copy Numbers in Stocks from Different DTUs

To estimate the satDNA copy number in *T. cruzi* stocks belonging to different DTUs, epimastigote forms of *T. cruzi* strains/clones (Dm28c, TcI; Y, TcII; INPA 3663, TcIII; INPA 4167, TcIV; Bug 2149, TcV; and CL-Brener, TcVI) were cultivated in liver infusion tryptose medium supplemented with 10% inactivated bovine fetal serum for 5 days at 28°C. Parasites were then pelleted, washed three times with phosphate-buffered saline, and counted in a Neubauer chamber. The GEB-seronegative samples were spiked with 10^4 and 10^2 parasites/mL, and DNA was extracted as previously described. Parasitic load was estimated as satDNA Q12 Q13 copies/μL, using the satDNA synthetic curve, as previously Q14 described. The qPCR experiments were performed in the same plate for all *T. cruzi* DTU DNA samples on duplicates. This experiment was performed from three separate cultures of each *T. cruzi* stock. To calculate the number of satDNA copies per parasite, we divided the number of satDNA copies/mL by the concentration of parasites/mL used as starting material for the DNA extraction and expressed the satDNA gene dosage of strains belonging to the different DTUs with respect to the dosage of TcI Dm 28 clone.

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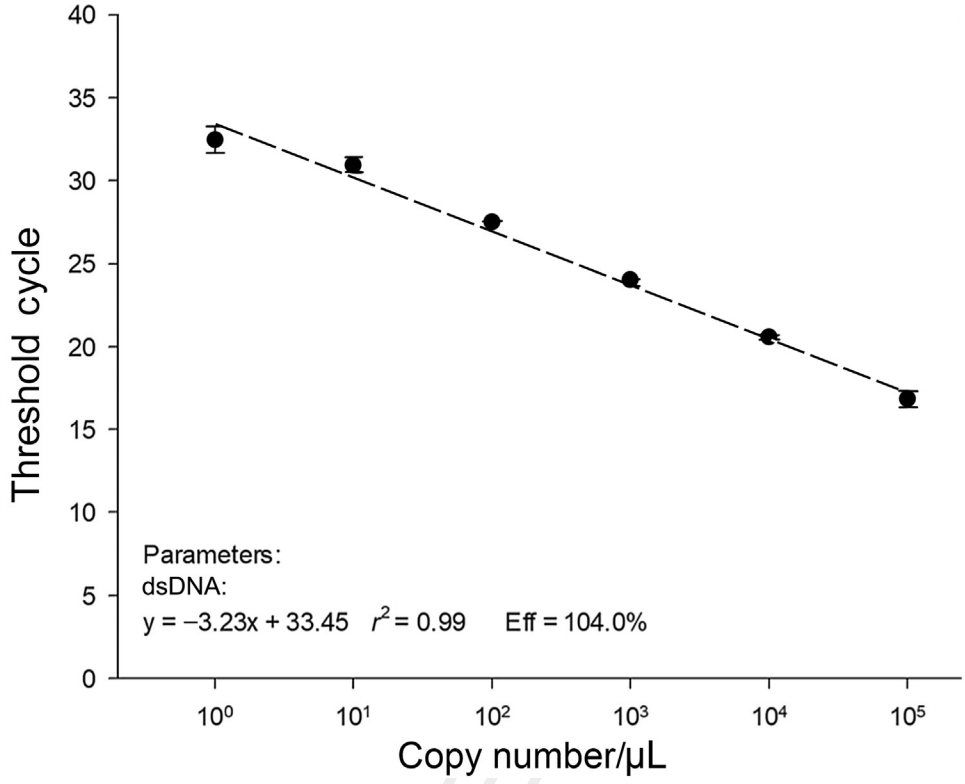

Figure 1 Quantitative curve of satellite DNA quantitative real-time PCR. Standard curve was generated by plotting the average threshold cycle values obtained by quantitative real-time PCR against 10-fold serial dilutions of the synthetic satellite DNA of *Trypanosoma cruzi* expressed in copy numbers/µL. dsDNA, double-stranded DNA.

Comparison of satDNA Copy Number in Epimastigote and Trypomastigote Forms from DM28c Clone (TcI)

Epimastigotes were obtained as described above, and cell-derived trypomastigote forms were obtained by using Vero cells. At first, to obtain metacyclic trypomastigotes with a high yield, *T. cruzi* epimastigotes (Dm28c) were allowed to differentiate under chemically defined conditions (TAU3AAG medium), as previously described.[19,20] Thereafter, Vero cell cultures were infected with metacyclic trypomastigotes, in a 10:1 parasite/host cell ratio. Infected cells were maintained at 37°C in a 5% carbon dioxide atmosphere. After 5 to 6 days, the supernatant was collected, centrifuged at 500 × *g* for 5 minutes, and allowed to stand at 37°C for 30 minutes for the migration of trypomastigotes to the supernatant. The supernatant was collected, and the trypomastigotes were pelleted, washed three times with phosphate-buffered saline, and counted in a Neubauer chamber.

DNA was extracted from 200 µL of parasite suspension containing 10, 100, and 10^4 epimastigote/mL or trypomastigote/mL, in parallel. The qPCR was performed as described above, targeting *T. cruzi* satDNA and internal amplification control. The fluorescent signal (threshold cycle value) was directly compared between epimastigote and trypomastigote forms. The reproducibility of DNA extraction and the absence of PCR inhibition were monitored by the internal amplification control. Each experiment was performed in biological triplicates for each parasite evolutive form (three independent cultures), and PCR was performed on experimental duplicates (two experimental points for each sample).

Assessment of 95% Limit of Detection Using Blood Spiked with Quantified Dm28c and CL-Brener Cells

The 95% limit of detection (LOD_{95}) of duplex satDNA expressed in copies/µL was calculated as the lowest parasitic load that gives ≥95% of qPCR detectable results, according to the Clinical and Laboratory Standards Institute guidelines.[12] The working panels consisted of eight replicates from GE blood of a pool of seronegative patients and spiked with *T. cruzi* cells to obtain 100, 1, 0.5, and 0.1 parasite equivalents/mL for both Dm 28c and CL-Brener clones. These artificial samples were purified and amplified by qPCR during five consecutive days using as standard curves both the synthetic DNA and DNA from blood spiked with cultured parasites. The LOD_{95} was determined by Probit regression analysis with the software RStudio Team, version 1.2.1335 (2015). Based on this model, the number of copies needed to achieve 95% probability of detection was estimated, along with the 95% CI.

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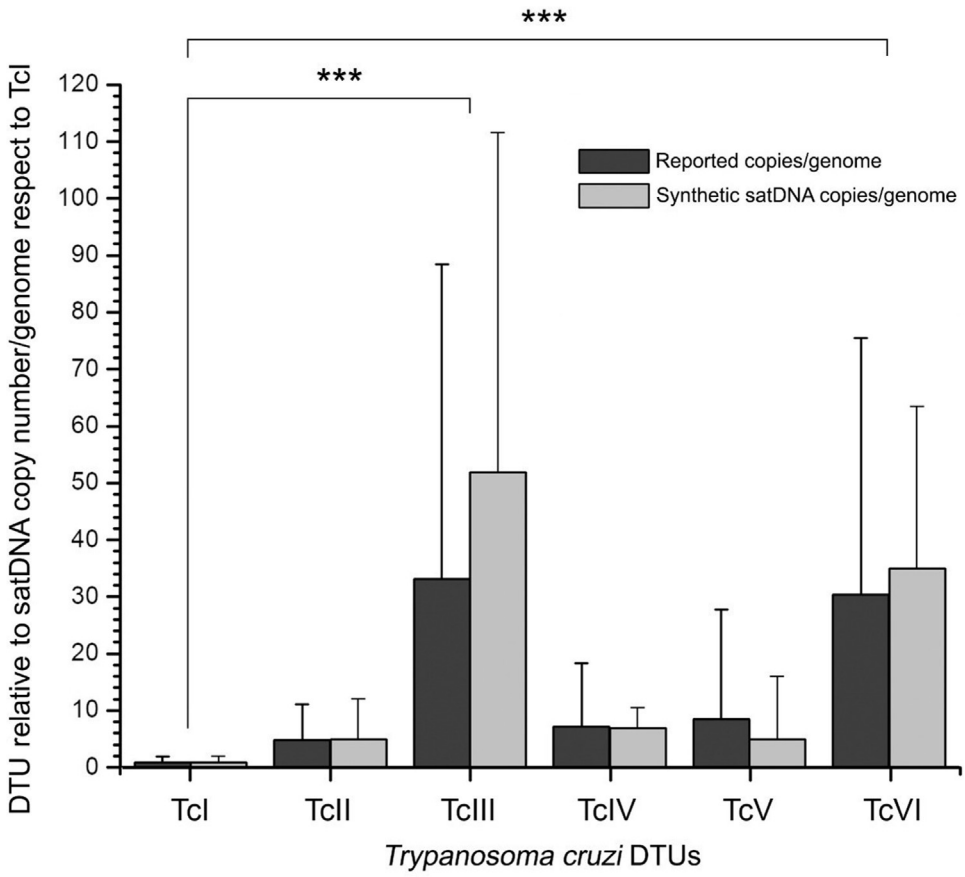

Figure 2 Relative satellite DNA (satDNA) copy number per genome in *Trypanosoma cruzi* strains representative of different discrete typing units (DTUs) with respect to Dm28c clone (TcI). **Black bars:** Relative satDNA gene dosage obtained from published data.[25,26] **Gray bars:** satDNA gene dosage estimated by quantitative real-time PCR against a standard curve based on the synthetic oligonucleotide. DTU II, Y; DTU III, INPA 3663; DTU IV, INPA 4167; DTU V, Bug 2149; DTU VI, CL-Brener. ***$P < 0.001$, one-way analysis of variance.

Patients and Blood Specimens

Peripheral blood samples from patients with CD were distributed into groups according to their geographic origin. Group 1 (G1) included 186 blood samples from 38 patients with oral transmission of CD infected at the outbreaks of Chacao (2007)[21] and Chichiriviche de la Costa (2009) in Venezuela,[22] collected at time of diagnosis and after treatment (covering for some of them 9 years of posttreatment follow-up); Group 2 (G2) included 19 blood samples from 19 patients with chronic CD from Brazil.[23]

G1 patients were infected with different TcI haplotypes[24] and G2 patients with *T. cruzi* II and/or *T. cruzi* VI strains.[23]

Samples from both patient groups were treated with one volume of guanidinium hydrochloride 6 mol/L, EDTA 0.2 mol/L, pH 8.00 stabilizing agent.[12]

To compare parasitic loads by using the synthetic oligonucleotide as standard, samples from G1 were quantified by using a standard curve produced with DNA extracted from blood spiked with known quantities of Silvio X10 clone (TcI), and samples from G2 were quantified by using a standard curve produced with DNA extracted from blood spiked with known quantities of Y strain parasites (TcII).

Statistical Analysis

A Bland-Altman analysis was used to estimate the agreement between the parasitic loads obtained in human blood spiked with known quantities of parasite cells using both types of standard curves for qPCR quantification.

Pearson's correlation analysis was conducted to compare the quantification done by using standard curves for quantification: i) serial dilutions of DNA obtained from seronegative blood spiked with parasite cells; and ii) synthetic satDNA.

For the comparison between the different parasitic load calculations (parasite equivalents/mL versus copies/μL), we proceeded to transform all values obtained for each patient's sample with z scores. The z score is the number of SDs by which the value of a raw score (ie, an observed value or a data point) is above or below the mean value of what is being measured. The z scores are calculated by subtracting the mean of the parasitic load values (within the same group of patients) and dividing that result by the SD of all parasitic loads measured, according to the formula: z score $= X - mean_{(X1—Xn)}/SD_{(X1—Xn)}$, where X is any parasitic load value on the assay and X1…Xn represents the aggregate measure of all parasitic loads.

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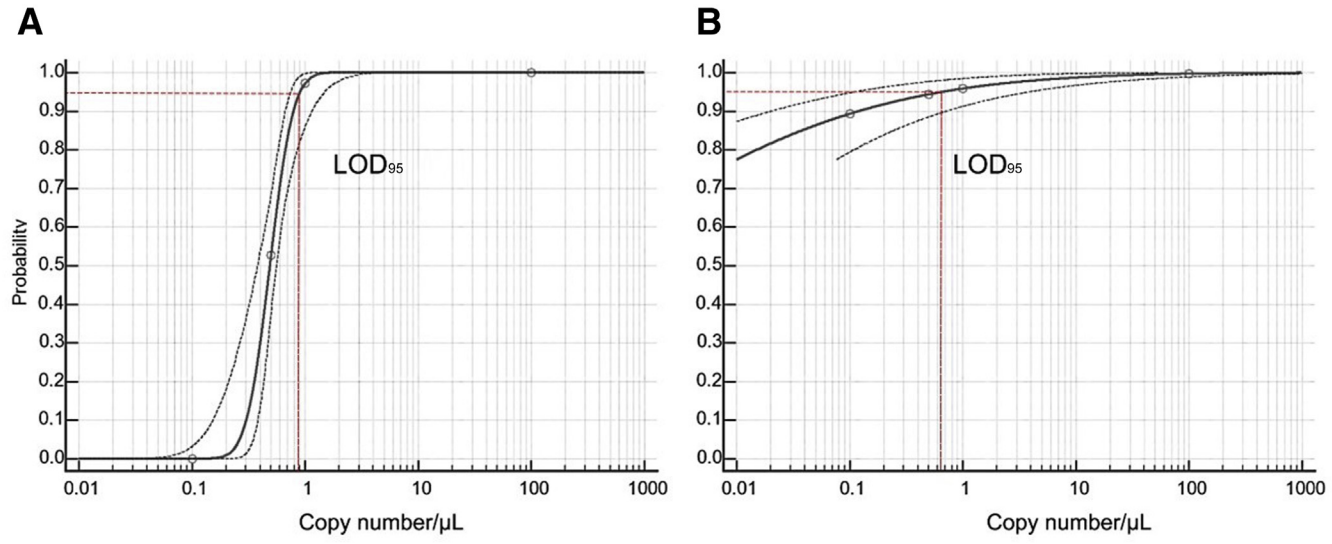

Figure 3 Probit regression (dose–response analysis) for the 95% limit of detection (LOD_{95}) of satellite DNA quantitative real-time PCR. **A:** LOD_{95} assay using Dm28c (discrete typing unit I); **B:** LOD_{95} assay using CL-Brener (discrete typing unit VI). The **red dotted border** corresponds to the estimate of LOD parameter with a 95% CI.

Results

Analytical Parameters of satDNA-Based qPCR Using Synthetic DNA as Standard Curve

To estimate the analytical sensitivity, linearity, and dynamic range of the qPCR using the synthetic satDNA as a standard, six serial dilutions of the oligonucleotide were tested in three independent assays, with technical duplicates per assay. The qPCR assay exhibited good linearity ($R^2 = 0.99$) with a dynamic range of detection from 1 to 10^5 copies/µL [F1] (Figure 1). The slope was −3.23, equivalent to a qPCR efficiency of 104%. According to the standard curves, the qPCR detection limit was determined as 1 copy/µL.

Copy Numbers of satDNA Sequence in Different *T. cruzi* Strains

The relative number of satDNA repeats was estimated with respect to those of the TcI Dm28 clone, by means of satDNA qPCR against a standard curve made of serial dilutions of the synthetic satDNA oligonucleotide, and compared with the relative satDNA gene dosage available from Vargas et al[25] and Souza et al.[26] Both strategies revealed similar differences among the *T. cruzi* strains used as representative of the different DTUs, except for the TcIII and TcVI strains [F2] (Figure 2). TcII (Y), TcIV (INPA 4167), and TcV (Bug 2149) stocks presented between four and eight times more dosage in satDNA repeats per genome than TcI. Stocks belonging to TcIII (INPA 3663) and TcVI (CL-Brener) exhibited between approximately 30 and 50 times more abundance in satDNA genes than the Dm28c clone (Figure 2).

Analysis of satDNA qPCR Based on Gene Dosage in Epimastigote and Trypomastigote Stages of Dm28c Strain (TcI)

The copy number of satDNA repeats obtained by using DNA from the Dm28c clone was corroborated in parallel experiments conducted with DNA obtained from the two life stages of the parasite (epimastigote and trypomastigote). The results indicated a similar gene dosage for these two life cycle stages of the parasite (Supplemental Figure S2).

Assessment of the LOD_{95} of satDNA qPCR Using the Synthetic DNA-Based Quantification Standard Curve

The LOD_{95} was estimated in DNA from human blood samples spiked with known quantities of parasites from Dm28c and CL-Brener cultures using the oligonucleotide as quantitation standard. The results of the Probit regression analysis established for the Dm28c stock of 0.903 copy numbers/µL (0.745 to 1.497) and for CL-Brener clone an LOD_{95} of 0.667 copy numbers/µL (CI, 0.113 to 3.927) when synthetic DNA was used as the standard for quantification (Figure 3). [F3]

Agreement between Parasitic Load Measurements in a Panel of Spiked Human Blood Using qPCR Standard Curves Based on Synthetic DNA or DNA Extracted from Blood Spiked with Parasite Cells

Bland-Altman analysis revealed a difference of −1.276 units in the parasitic load measurements performed by using standard curve spiked blood or synthetic DNA (Figure 4). [F4] This difference of means, being nonzero, indicates that on average, the quantification approach using satDNA copy

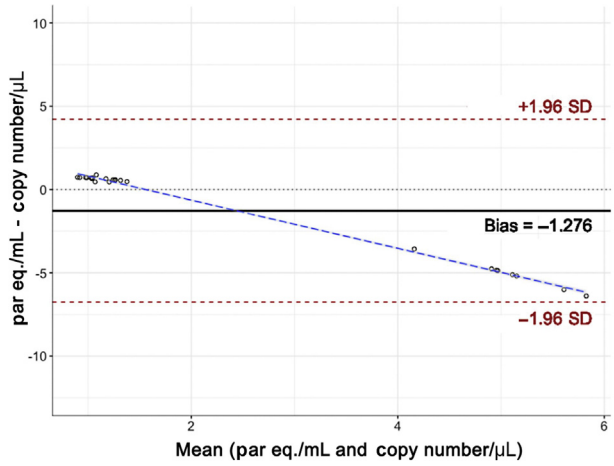

Figure 4 Agreement of parasitic load measurements in samples spiked with *Trypanosoma cruzi* using spiked blood or synthetic DNA, by means of Bland-Altman concordance analysis. par.eq/mL, parasite equivalents/mL per of blood.

numbers measured 1.276 more units than the quantification approach using parasite equivalents.

Comparison of *T. cruzi* Loads in Clinical Samples Using Both Types of Standard Curve

A comparative study of parasitic load quantification using standard curves according to the novel synthetic satDNA and gDNA from spiked blood was conducted in pretreatment and/or posttreatment blood samples collected from G1 and G2 patients (Table 2).

A comparative analysis of the bloodstream *T. cruzi* loads obtained for each clinical group was performed by using two standard curves for quantification: serial dilutions of DNA obtained from seronegative blood spiked with parasite cells and synthetic satDNA. The Pearson's correlation analysis between both measurements was 0.995 for G1 and 0.884 for G2 (Figure 5, A and B, respectively). The box plots for each group of patients shown in Figure 5C indicate the differences between the measurements of parasitic loads using both standard curves. These differences were close to the level of significance for G1 but not for G2.

Use of Parasitic Loads to Follow Up Patients with CD under Treatment

We compared the parasitic loads measured by using both quantification approaches for G1 patients followed up during and after treatment with antiparasitic drugs (Figure 6). In general, a parallel trend in fluctuations of the parasitic loads during follow-up was observed when either of the standard curves (based on parasite equivalents or copy numbers) were used.

Discussion

Equivalence of Copy Numbers and Parasite Equivalents

In CD, the parasitic load quantification by qPCR has opened possibilities to monitor etiologic treatment failure in patients with CD[27–30] and disease reactivation in immunosuppressed patients, as well as in heart (or other organ) transplant recipients and *T. cruzi*–HIV coinfection, CD patients with autoimmune diseases or under oncologic chemotherapies, and de novo infection in recipients of organs from *T. cruzi*–infected donors.[31] In addition, in multicenter trials, qPCR assays have long been used to compare parasitic loads between patient cohorts from different countries or regions.[14] Since the last decade, several protocols were reported, using standard curves from different *T. cruzi* strains/DTUs as references.[6–8,13–15] However, due to the heterogeneity between *T. cruzi* strains/DTU genomes, the establishment of a unique standard curve is challenging. In general, those studies used different standard curves, employing cultured parasite isolates with the highest prevalence in each region that allowed a more accurate quantification for most patient samples, assuming that they were infected by populations belonging to a single DTU. Nevertheless, this approach could generate a bias for the direct comparison of parasitic loads among clinical samples from

Table 2 Parasitic Load Quantification in Blood Samples from Patient Groups Using Synthetic satDNA and Artificial Blood Samples Spiked with Cultured Parasites

	Group 1		Group 2	
Variable	par.eq/mL	Copy number/µL	par.eq/mL	Copy number/µL
Total samples	186	186	19	19
Total positive samples	112	111	15	14
Quantifiable positive	104	59	1	14
Nondetectable results	74	75	4	5
Median [Q1-Q3]	343.83 [7.12-2362.54]	1.00 [0.01-13.69]	0.12 [0.09-0.71]	8.72 [5.02-19.25]

Quantifiable positive samples are samples that exhibited parasitic loads above the lower limit of detection of the method, taking into account the discrete typing unit prevalent in the tested patient population. Group 1, TcI orally infected patients from Venezuela. Quantifiable positive cases cutline: 1 parasite equivalent/mL of blood (par.eq/mL), lower value of linear reportable range of quantitative real-time PCR for TcI.[13] Group 2, TcII and TcVI chronically infected patients from Brazil. Quantifiable positive cases cutline: 1.53 par eq./mL, limit of quantification of multiplex Real-Time PCR assay using TaqMan Probes for *Trypanosoma cruzi* satellite DNA (satDNA) in blood samples.[13,15] [Q1-Q3], first and third quartile.

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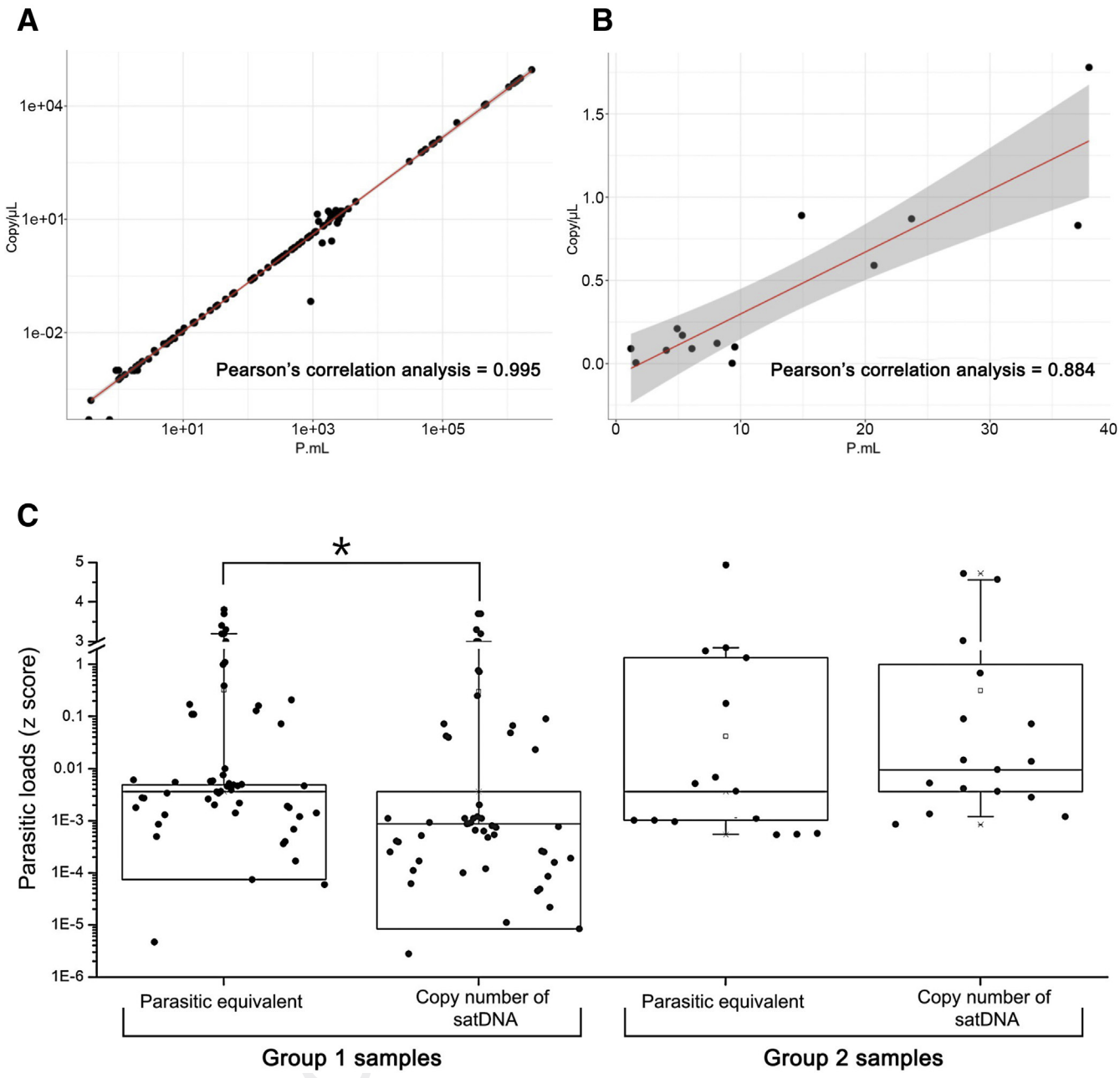

Figure 5 Comparison of *Trypanosoma cruzi* loads in clinical samples using standard curves based on spiked blood with parasite cells or synthetic DNA. **A:** Pearson's correlation analysis for patients in Group 1. **B:** Pearson's correlation analysis for patients in Group 2. **C:** Comparative box plots between the two quantitative real-time PCR–based quantification approaches in both groups of patient samples. To compare the different measurements of parasitic load, the data were normalized by calculating the z scores. The gray shaded area corresponds to 95% CI according to the statistical test. *$P = 0.05$. satDNA, satellite DNA.

different patients, even within the same geographical region, as there may exist an overlapping presence of different *T. cruzi* strains/DTUs with variable genome content and thus present differences in the target gene copy numbers in the same cohort of patients and even in a single individual.[16–18]

To overcome these limitations, the current study proposes the use of a synthetic DNA standard curve that allows comparative quantification of parasitic loads independently of the parasite genotype by expressing the parasitic burden as satDNA copy numbers/µL of a tested sample. This DNA standard sequence was conserved among satDNA sequences from strains belonging to the six *T. cruzi* DTUs, available at the GenBank (Supplemental Figure S1) with a very low proportion of single nucleotide polymorphisms within the regions recognized by the primers. This qPCR quantification method was evaluated with blood samples from patients with CD from two different geographical regions and infected with different *T. cruzi* DTUs.[23,24]

The double-stranded satDNA-based synthetic standard curve presented a linearity of six logs, ranging from 10^5 up

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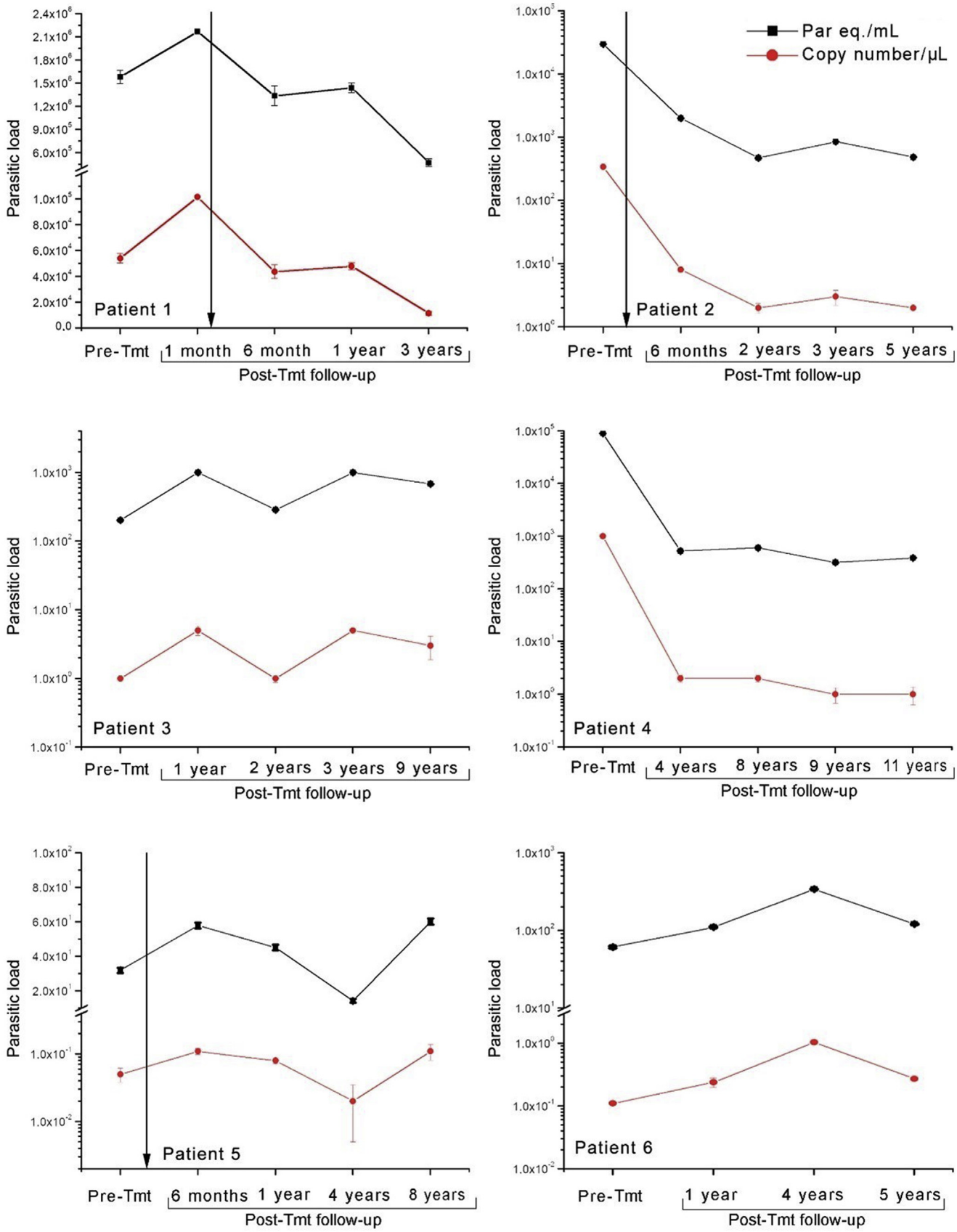

Figure 6 Evolution of parasitic loads during treatment follow-up of a convenience series of patients with oral transmission of Chagas disease patients from Group 1. The **arrows** indicate the end of treatment for 60 days with benznidazole in patients 1, 2, and 5. Post-Tmt, posttreatment; Pre-Tmt, pretreatment.

to 1 copy/μL, with a qPCR efficiency of 104%. This dynamic extension comprises the expected range of amplification for patients with acute CD as well as for those with chronic CD, and it is in agreement with standard curves previously reported for other DNA pathogens such as hepatitis A and E viruses,[32] human herpes virus 1 and 2,[33] and *Plasmodium falciparum*.[34] When the satDNA copy number was quantified in representative strain/clones from DTUs I-VI, significant differences were observed between TcI and TcIII, and TcI and TcVI. The Dm28c (TcI) clone presented the lowest satDNA copy number of all tested parasite DNA stocks, including strains belonging to TcII and TcVI, as previously reported by the relative quantification of satDNA copies compared with a single-copy housekeeping gene.[7]

Most of the current qPCR protocols for *T. cruzi* quantification in blood samples recommend the standard curve preparation using DNA extracted from a pool of epimastigotes, which is more feasible to obtain in axenic cultivation. However, the parasite form circulating in blood is mostly the trypomastigote stage. To confirm that no significant difference in satDNA copy numbers exists between both life cycle stages of the same strain, we performed a direct comparison between these two forms using DNA extracted from three concentrations (10,000, 100, and 10 parasite equivalents/mL) and the relative threshold cycle values of axenic epimastigotes and metacyclic trypomastigotes of a representative stock of TcI. No statistical differences were observed at any tested concentration, corroborating no variation in satDNA copy numbers to both extracellular life stages of the parasite and confirming the accuracy of standard curves produced with DNA obtained from epimastigote forms.

Usefulness of Measuring Parasitic Loads in Specimens from Patients Infected with Different DTUs

It is worth noting that the sequence of satDNA used to produce the synthetic DNA is conserved in different clones and strains of *T. cruzi*, belonging to different DTUs.[35,36] In previous studies, when the inclusivity and analytical sensitivity of satDNA-based qPCR were estimated by using gDNA from different parasite strains, different values of sensitivity were detected, which correlated with the various *Satellite* gene dosage.[13,15] Furthermore, the limits of quantification of this qPCR varied depending on the strain.[13,15]

This scenario led to recommendations for using different standard curves for quantification of parasitic loads.[14,15] However, when the synthetic oligonucleotide was used in the standard curve, the LOD_{95} values of the qPCR measured by using gDNA extracted from blood spiked with DM28c (TcI) or CL-Brener (TcVI) parasite cells, which harbor a different satDNA gene dosage (Figure 2), resulted in similar findings: 903 copy numbers/mL (745 to 1497) and 667 copy numbers/mL (113 to 3927), respectively. This outcome suggests that the synthetic DNA allows direct comparison of qPCR loads in samples infected with different DTUs. Accordingly, the qPCR-based quantification strategy presented herein will allow direct comparison of parasitic loads expressed in copy numbers among patients from various geographical regions infected with different *T. cruzi* genotypes, which will be of true benefit for meta-analysis of findings obtained in clinical trials. If the DTU of a given clinical sample could be identified, an equivalence between the dosage in copy numbers of the satDNA repeats and parasite genome equivalents per unit of sample volume could be estimated if the satDNA copy dosage per parasite cell is known for the DTU under study. Nevertheless, it must be taken into account that even within a specific DTU, intra-DTU variability in satDNA copy numbers exists among strains.[25,26,35,36] In addition, a given patient may be infected with multiple strains belonging to different DTUs.

The incorporation of qPCR standard curves employing the synthetic DNA, as well as others that should be designed for qPCR assays using other molecular targets (eg, kinetoplastid DNA), would be of valuable support in multicentric phase 3 clinical trials in which outcomes are based on parasitic DNA load as a surrogate marker of treatment response. Furthermore, this approach could be applied in clinical practice to follow-up parasitic loads of patients with risk of CD reactivation due to immunosuppression conditions and in experimental models working with *T. cruzi* strains belonging to different genotypes.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2021.01.007*.

Author Contributions

O.C.M. and A.G.S. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and accuracy of data analysis. O.C.M. and A.G.S. conceived and designed the study; O.C.M. and S.G.N. designed the synthetic oligonucleotide; C.A.D.T. and L.R.Q.S. conducted the experiments related to synthesis, cloning, and analysis of synthetic oligonucleotide sequence; A.M.-C., S.G.N., and S.A. conducted the experiments related to analytical validation of the qPCR; A.N.B. and D.B.Z. admitted and treated patients with orally transmitted CD and obtained samples for qPCR, which were tested by A.M.-C.; S.L.R.Q. and B.C.

admitted and followed up Brazilian patients; A.G.S., C.B., and O.C.M. supervised analysis and interpretation of results; A.M.-C., A.G.S., and O.T. wrote the original draft and the revised manuscript; and O.T., C.B. and A.G.S. gave financial support to the work. All authors approved the final version of the manuscript.

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Supplemental Figure S1 Alignment of satellite DNA (satDNA) primers and probe with satellite repeats from different *Trypanosoma cruzi* strains covering the six discrete typing units. The gray boxes correspond to the regions in which the oligonucleotides and the probe are located in the microsatellite sequence.

Supplemental Figure S2 Comparison of threshold cycles obtained by means of satellite DNA quantitative real-time PCR using paired quantities of epimastigote and trypomastigote forms of the *Trypanosoma cruzi* Dm28c clone.