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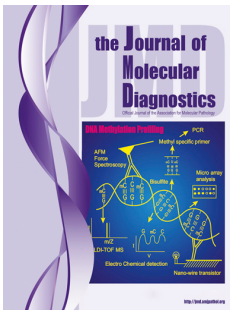

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Towards the establishment of a single standard curve for quantification of *Trypanosoma cruzi* natural populations using a synthetic *satellite* unit DNA sequence

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Short Running Title: Synthetic *T. cruzi satDNA* qPCR standard

Abstract

Accurate diagnostics tools and surrogate markers of parasitological response to treatment are priority needs for management of 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 (*sat*DNA) repeat unit as standard for quantification of *T. cruzi* loads in clinical samples, independently of the parasite strain. Probit regression analysis established for Dm28c (Tc I) and CL-Brener (Tc VI) stocks similar LOD_{95} values (0.903 (0.745-1.497) and 0.667 (CI 0.113-3.927) copy numbers/µL, respectively), when synthetic DNA was the standard for quantification, thus allowing direct comparison of loads in samples infected with different DTUs. This standard curve was evaluated in 205 samples from 38 acute oral and 19 chronic CD patients from different geographical areas infected with different genotypes, including samples obtained during treatment follow-up, and 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 III clinical trials, to follow-up patients under treatment or at risk of reactivation and in experimental models using different parasite strains.

Keywords: Real Time PCR, *Trypanosoma cruzi*, discrete typing units, parasitic load quantification, molecular diagnosis, synthetic oligonucleotide, satellite sequence.

Introduction

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 9,000 newborns becoming infected during gestation. Chagas disease 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 non-endemic countries [2, 3].

Accurate diagnostics tools and surrogate markers of parasitological response to treatment are priorities in CD research and development [4]. In order to develop an accurate laboratory tool for diagnosis and treatment follow-up of CD patients, several difficulties have to be addressed, such as the low and intermittent parasitic burden during the chronic phase of infection and the parasite genotype diversity, because *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 carried out prior to be confident to use this laboratory tool in clinical trials and practice [12-15]. So 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, 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 re-testing of clinical samples by the different laboratories involved [20, 23]. However, mostly in multicenter clinical trials that covered patients´ cohorts from different countries where different *T. cruzi* genotypes prevail, difficulties were encountered regarding the trueness and precision of quantitative measurements. 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 sequence [24] varies among parasite strains [7, 12, 14, 24-26]. This has led to the need of quantitative standard curves built up with DNA isolated from parasite stocks representative of the discrete typing units (DTUs) prevailing in each clinical setting and geographical area [13-15]. However, this makes difficult performing 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 have designed and evaluated a synthetic oligonucleotide with the *Satellite* DNA sequence repeat to be used as 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 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 from 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, 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, 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 M Guanidinium Hydrochloride / 0.2 M 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., 2013 [13].

To build the standard curves for quantification of parasitic loads, DNA from spiked blood samples were 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 *satellite* DNA 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 (Supplementary Figure 1). A 166-bp region of *T. cruzi* nuclear *satellite* DNA 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 using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), according to the manufacturer`s instructions. The PCR product was cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, USA), according to the manufacturer`s instructions. Three positive clones were isolated and submitted to DNA sequencing using the Applied Biosystems ABI 3730xl 96-capillary DNA analyzer, at the DNA sequencing Platform RPT01A from the Oswaldo Cruz Foundation, employing the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Sequences were analyzed using Sequence Scanner v1.0 (Applied Biosystems, Foster City, USA) and aligned against a Nuclear *Satellite* DNA reference sequence (GenBank AY520087.1 (https://www.ncbi.nlm.nih.gov/nuccore/) - *Trypanosoma cruzi* clone YA05 *satellite* sequence, from Y strain) with Mega Software v4.0.2 software. A sequence containing the majoritary SNPs among the cloned sequences was chosen to produce the synthetic oligonucleotide (GBlock Gene Fragments, Integrated DNA Technologies, Coralville, USA) (Table 1). Its concentration was estimated using a

ND 2000 spectrophotometer (Nanodrop) and the copy number determined using the DNA Copy Number Calculator (ThermoFisher Scientific, Waltham, USA) considering the DNA fragment length and the Molar mass per base pair.

Duplex Real-Time qPCR Procedures

The standardized *Satellite* DNA (*Sat*DNA) qPCR assay used Cruzi 1 - Cruzi 2 primers and Cruzi 3 probe reported by Piron and coworkers [6] and the Exogenous Internal Amplification Control (IAC) reported by Duffy and coworkers [7]. The reaction was performed with 5 µL of re-suspended DNA, using FastStart Universal Probe Master Mix (Roche Diagnostics GmbHCorp. Mannheim, Germany) in a final volume of 20 uL. 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 using Rotor-Gene 6000 (Corbett Life Science, Cambridgeshire, United Kingdom) or ABI7500 (Applied Biosystems, Foster City, CA) devices.

Construction of Standard curves for qPCR quantification.

To carry out comparative qPCR experiments using the novel synthetic SatDNA sequence as standard and genomic DNA isolated from cultured parasites, two types of standard curves were constructed, as follows:

Standard curve using blood spiked with cultured parasites. This type of standard curve was plotted with total DNA obtained from a seronegative-GEB sample spiked with 10^5 parasite equivalents per milliliter of blood (par.eq/mL) and 1/10 serial dilutions were done using as a matrix, total DNA obtained from a pool of blood samples from seronegative individuals, as reported in previous studies.[13]

Trypanosoma cruzi DTU I (Dm 28c clone) and DTU VI (CL-Brener clone) (gDNA) genomic DNA-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 per microliter (copies/µL) and 1/10 serial dilutions were done 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

In order 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, CL-Brener: TcVI) were cultivated in LIT medium supplemented with 10% inactivated Bovine Fetal Serum (BFS) for 5 days, at 28°C. After that, parasites were pelleted, washed three times with PBS and counted at a Neubauer chamber. The GEB-seronegative samples were spiked with 10^4 and 10^2 parasites/mL and DNA was extracted as previously described. Parasite load was estimated as satDNA copies/µL, using the satDNA synthetic curve, as previously described. The qPCR experiments were carried out in the same plate for all *T. cruzi* DTU DNA samples on duplicates. This experiment was done 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 Tc I Dm 28 clone.

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 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 37ºC in a 5% CO_2 atmosphere. After 5–6 days, the supernatant was collected, centrifuged at 500 ×g for 5 min, and allowed to stand at 37ºC for 30 min for the migration of trypomastigotes to the supernatant. The supernatant was collected and the trypomastigotes were pelleted, washed three times with PBS and counted at 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 IAC. The fluorescent signal (Ct value) was directly compared between epimastigote and trypomastigote forms. The reproducibility of DNA extraction and the absence of PCR inhibition were monitored by the IAC amplification. Each experiment was performed in biological triplicates for each parasite evolutive form (3 independent cultures) and PCR was done on experimental duplicates (two experimental points for each sample).

Assessment of Limit of Detection (LOD 95%) using blood spiked with quantified Dm28c and Cl-Brener cells.

The Limit of Detection (LOD) 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% confidence interval (CI).

Patients and Blood Specimens

Peripheral blood samples from CD patients were distributed into groups according to their geographic origin, as follows: Group 1 (G1) included 186 blood samples from 38 oral CD patients 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 post-treatment follow-up) and Group 2: 19 blood samples from 19 chronic CD patients 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 patients' groups were treated with one volume of Guanidinium Hydrochloride 6M, EDTA 0.2 M, pH 8.00 stabilizing agent.[12]

. To compare parasitic loads using the synthetic oligonucleotide as standard, samples from G1 were quantified 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 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 curve for qPCR quantification

Pearson´s correlation analysis was done to compare the quantification done using as 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 (par eq./mL vs. copies / uL), we proceeded to transform all values obtained for each patient`s sample with Z-scores.The Z-score is the number of standard deviations by which the value of a raw score (that is, 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 standard deviation (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.

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 (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 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 Tc III and Tc VI strains (Figure 2). TcII (Y), TcIV (INPA 4167) and TcV (Bug 2149) stocks presented between 4-8 times more dosage in satDNA repeats per genome than Tc I. Stocks belonging to TcIII (INPA 3663) and TcVI (CL-Brener) showed 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 using DNA from the Dm28c clone was corroborated in parallel experiments carried out 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 (Supplementary Figure 2).

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 (0.745-1.497) and for CL-Brener clone a $LOD_{95\%}$ of 0.667 copy numbers/µL (CI 0.113-3.927) when synthetic DNA was used as the standard for quantification (Figure 3).

Agreement between parasitic loads 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 showed a difference of -1.276 units in the parasitic loads measurements done using as standard curve spiked blood or the synthetic DNA (Figure 4). This difference of means, being non-zero, indicates that on average, the quantification approach using satDNA copy 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 using the novel synthetic satDNA and genomic DNA from spiked blood was carried out in pre-treatment and/or post-treatment blood samples collected from G1 and G2 groups of patients (Table 2).

A comparative analysis of the bloodstream *T. cruzi* loads obtained for each clinical group was carried out using two standard curves for quantification: i) serial dilutions of DNA obtained from seronegative blood spiked with parasite cells and ii) synthetic satDNA. The Pearson´s correlation analysis between both measurements was 0.995 for Group 1 and 0.884 for Group 2 (Figures 5 A and B, respectively). The box plots for each group of patients shown in Figure 5C indicated the differences between the measurements of parasite loads using both standard curves. These differences were close to the level of significance for Group 1 but not for Group 2.

Use of parasitic loads to follow-up CD patients under treatment

We compared the parasitic loads measured 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 the standard curves (based on parasite equivalents or copy numbers) were employed.

Discussion

Equivalence of copy numbers and parasite equivalents.

In Chagas disease, the parasitic load quantification by qPCR has opened possibilities to monitor etiological treatment failure in CD patients [27-30], disease reactivation in immunosuppressed patients, as well as in heart (or other organs) transplant recipients and *T*. *cruzi*-HIV coinfection, CD patients with autoimmune diseases or under oncologic chemotherapies and *de novo* infection in receptors of organs from *T. cruzi* infected donors[31]. In addition, in multicenter trials, quantitative real time PCR assays have long been used to compare parasitic loads between patients` 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/DTUs genomes, the establishment of a unique standard curve is challenging. In general, those studies employed different standard curves, using cultured parasite isolates with the highest prevalence in each region that allowed a

more accurate quantification for most patients` 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 different patients, even within a same geographical region, since it may exist an overlapping presence of different *T. cruzi* strains/DTUs with variable genome content and thus presenting differences in the target gene copy numbers in the same cohort of patients and even in a same individual.[16-18]

To overcome these limitations, this work proposes the use of a synthetic DNA standard curve that allows comparative quantification of parasitic loads independently of the parasite genotype by means of expressing the parasitic burden as satDNA copy numbers/µL of tested sample. This DNA standard sequence was conserved among satDNA sequences from strains belonging to the six *T. cruzi* DTUs, available at the Genbank (Supplementary Figure 1) with a very low proportion of SNPs within the regions recognized by the primers.

This qPCR quantification method was evaluated with blood samples from CD patients 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 to 1 copy/µL, with a qPCR efficiency of 104%. This dynamic extension comprises the expected range of amplification for acute as well as for chronic CD patients, 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 *satellite* DNA copies in comparison to a single copy housekeeping gene [7].

Most of 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. In order to confirm that there is no significant difference in satDNA copy numbers between both life cycle stages of a same strain, we performed a direct comparison between these two forms using DNA extracted from three concentrations (10000, 100 and 10 parasite equivalents/mL) and the relative Ct 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 to note 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 works, when the inclusivity and analytical sensitivity of satDNA-based qPCR was estimated using gDNA from different parasite strains, different values of sensitivity were detected, which correlated with the different *Satellite* gene dosage[13,15]. Furthermore, the limits of quantification of this qPCR varied depending on the strain. [13,15]

This led to recommend the use of 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 using gDNA extracted from blood spiked with DM28c (TcI) or CL-Brener (Tc VI) parasite cells, which harbor a different satellite DNA gene dosage (Figure 2) resulted similar; 903 copy numbers/mL (745-1,497) and 667 copy numbers/mL (113-3,927), respectively, which 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 different 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] and 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, such as 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 the 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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Author contributions

OM and AGS 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. OM and AGS conceived and designed the study. OM and SGN designed the synthetic oligonucleotide; CADT and LRQ carried out

experiments related to synthesis, cloning and sanalysis of synthetic oligonucleotide sequence. AMC, SGN and SA carried out experiments related to analytical validation of the qPCR. ANB and DBZ admitted and treated oral CD patients and obtained samples for qPCR, which we tested by AMC. SLRQ and BC admitted and followed-up Brazilian patients. AGS, CB and OM supervised analysis and interpretation of results. AMC, AGS and OT wrote the original draft and the revised manuscript. OT, CB and AGS gave financial support to the work. All authors approved the final version of the manuscript.

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FIGURE LEGENGS

Figure 1. Quantitative curve of satDNA qPCR. Standard curve was generated by plotting the average Ct values obtained by qPCR against ten-fold serial dilutions of the synthetic satDNA of *Trypanosoma cruzi* expressed in copy numbers/ul.

Figure 2. Relative satDNA copy number per genome in *T. cruzi* strains representative of different DTUs with respect to Dm28c clone (Tc I). Black bars: Relative SatDNA gene dosage obtained from published data[25,26]. Grey bars: SatDNA gene dosage estimated by qPCR 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.

Figure 3. Probit regression (Dose-Response analysis) for the Limit of Detection ($LOD_{95\%}$) of satDNA qPCR. A) LOD assay using Dm28c (DTU I); B) LOD assay using CL-Brener (DTU VI). The red dotted border corresponds to the estimate of LOD parameter with a 95% confidence interval.

Figure 4. Agreement of parasitic load measurements in samples spiked with *T.cruzi* using spiked blood or synthetic DNA, by means of Bland-Altman concordance analysis.

Figure 5. Comparison of T. 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 of Group 1; B:) Pearson´s correlation analysis for patients of Group 2; C:) Comparative box-plots between the two qPCR-based quantification approaches in both groups of patients` samples. To compare the different measurements of parasite load, the data were normalized by calculating the z-scores. *P=0.05. The grey shaded area corresponds to 95% confidence interval according to the statistical test.

Figure 6. Evolution of parasitic loads during treatment follow-up of a convenience series of oral CD patients from Group 1 (Tmt: treatment). The arrows indicate the end of treatment for 60 days with Benznidazole in patients 1, 2 and 5.

Table 1. Sequences of *T. 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´AGTCGGCTGATCGTTTTCG AGCGGCTGCTACATCACACGT TGTGGTCTAAATTTTTGTTTC CAATTATGAATGGTGGGAGTC AGAGGCACTCTCTGTCACTAT CTGTTTGCGTGTTCACACACT GGACACCAAACAACCCTGAAT TATCCGCTGCTTGGAGGAATT 3´	166	XM_805618.1	*Trypanosoma cruzi* strain Cl-Brener hypothetical protein Tc00.1047053508097.10 partial mRNA	98%	97%	$3x10^{-74}$
Exogenous Internal Positive Control (IAC)	5´ACCGTCATGGAACAGCACG TACCGATTTATAAGATTGCTG GAGAAATGACTGGATTTGGAG CATCTGTTCTTGAAGGTGTTT TAGCTTTCGTCTTGGTTTATA CTGTGTTCACGGCTAGCGATC CCAGACGTGGGCTACCTTTAG CAGTGGGACCTATATTTATAG GGTTTGTTGCGGGAG 3´	181	NM_114612.3	*Arabidopsis thaliana* putative aquaporin TIP5-1 mRNA, complete cds	100%	100%	$2x10^{-88}$

Sequence ID's available from https://www.ncbi.nlm.nih.gov/.

Table 2. Parasitic load quantification in blood samples from different patients´ groups using synthetic satDNA and artificial blood samples spiked with cultured parasites.

	Group 1		Group 2	
	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
Non-Detectable 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 showed parasitic loads above the lower limit of detection of the method taking into account the DTU prevalent in the tested patient population. [Q1-Q3]: First and Third Quartile. G1: Tc I orally infected patients from Venezuela. Quantifiable positives cases cutline: 1 par eq./mL, lower value of linear reportable range of qPCR for Tc I.[13] G2: Tc II and TcVI chronically infected patients from Brazil. Quantifiable positives cases cutline: 1.53 par eq./mL, limit of quantification of multiplex Real-Time PCR assay using TaqMan Probes for *T. cruzi* SatDNA in Blood Samples[13, 15]