Monitoring the parasite load in chronic Chagas disease patients: comparison between blood culture and quantitative real time PCR

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Abstract

Background

Despite the improvements in diagnostic tools for detection of Trypanosoma cruzi in human blood samples, the isolation of parasite from bloodstream in the chronic phase of Chagas disease is challenging. Thus, there is an increasing interest in the development of strategies that allow an accurate monitoring of the parasite load in bloodstream of Chagas disease patients. Given that, the comparison of a classical diagnostic method such as blood culture and multiplex quantitative real-time PCR (qPCR) was few explored so far. Therefore, this study aimed to compare the detection and quantification of T. cruzi load in the circulating blood of patients with chronic Chagas disease, using blood culture and qPCR techniques.

Methods/Principal findings

The multiplex real-time quantitative PCR assay (qPCR) based on TaqMan technology was evaluated in 135 blood samples from 91 patients with chronic Chagas disease presenting indeterminate (asymptomatic, n = 23) and cardiac (chronic cardiomyopathy, n = 68) forms, in comparison with the classical blood culture (BC) technique. The total positivity of qPCR and BC was 58.5% and 49.6%, respectively. The median parasite load of all positive patients was 1.18 [0.39–4.23] par. eq./mL, ranging from 0.01 to 116.10 par. eq./mL. We did not find significant differences between T. cruzi load with age and distinct clinical manifestations of patients.

Conclusions/Significance

Our data suggest that qPCR can be an auxiliary tool for studies that require T. cruzi isolation from the bloodstream of patients with chronic Chagas disease, after the establishment of a parasite load cut-off that guarantees a relative success rate of parasite isolation using BC technique.
Introduction

The diagnosis of Trypanosoma cruzi infection should be carried out using different methodologies, depending on the stage of the disease. In the acute phase of the infection, the parasitemia is high in the peripheral circulation and the diagnosis of Chagas disease can be performed by direct examination of the blood. In contrast, in the chronic phase of the disease the parasitemia is subpatent, transient and depends on the immune response of each patient [1]. Current methods available for parasitological and serological diagnosis have limitations in sensitivity and specificity, especially when applied for the diagnosis in chronic phase of the disease. The major limitation of serological methods is the low specificity, due to cross-reactions with other trypanosomatids present in endemic areas such as Leishmania sp. and Trypanosoma rangeli [2–4]. Another difficulty is that following anti-T. cruzi specific treatment, serological methods remain positive for several years. In the chronic phase of the disease, the post treatment serological reversion is less than 10%, which impairs the efficacy of therapeutic evaluation [5–10].

Indirect parasitological diagnostic methods such as xenodiagnosis and blood culture (BC) depend on the presence of at least one intact trypomastigote form for its growth in culture medium. The results of these methods can take up to 120 days and still be doubtful [6, 11–13]. Negative results of BC and/or xenodiagnosis may be due to low parasitemia observed in the chronic phase of Chagas disease and do not rules out the possibility of infection. On the other hand, a positive test has an absolute diagnostic value [14]. In individuals with inconclusive serology, BC is an important tool for identifying T. cruzi, and when positive it is possible the parasite isolation for biological, biochemical and molecular studies [15,16].

The main technique that has been tested for the research of T. cruzi directly in the blood of chronic-affected patients is the conventional PCR, based on the use of synthetic oligonucleotides that amplify specific DNA sequences of the parasite, presenting high sensitivity and promising results, although it is not feasible for a quantitative evaluation [17–20]. The difficulties in the diagnosis of Chagas disease in chronic phase justify the interest and the necessity of implementation of a direct and more sensitive method that allows monitoring the presence of the parasite and confirming the etiology of the disease.

In the last decade, the methodology used for the detection of genes and specific sequences of T. cruzi has been improved with the development of different real-time PCR systems. An automated quantitative approach based on the use of fluorogenic probes (TaqMan) or fluorescent dyes with DNA affinity (SYBRGreen) has been useful for demonstrating the absolute levels of T. cruzi circulating in infected individuals. This methodology represents a major advance in molecular diagnostic methods and gives support to research laboratories, particularly facilitating the quantification of DNA or RNA fragments in different biological samples and, capable of accurately estimates T. cruzi parasite load of patients in chronic phase of Chagas disease [21–34]. In addition, it allows the monitoring of disease progression, evaluation of parasitemia in response to specific treatment, congenital infection and early detection of reactivation [21–34]. The qPCR is a more advantageous methodology when compared to conventional PCR and BC, since it presents higher sensitivity and earlier outcome for confirming the infection. Furthermore, it evaluates and quantifies the parasite load, being useful for medical decision regarding the introduction or not of specific therapeutic against T. cruzi infection. The aim of this study was to evaluate the qPCR (TaqMan system) and blood culture strategies for detecting T. cruzi load in asymptomatic and cardiac patients with chronic Chagas disease without previous etiological treatment, since the comparison of classical parasitological method BC with qPCR was few explored. Genotyping was performed to determine the genetic profile of T. cruzi in newly isolated strains of infected patients.
Materials and methods

Study population

This study included 91 patients in the chronic phase of Chagas disease from different endemic regions of the state of Minas Gerais (Southern Brazil). All patients were adults and had at least two positive conventional serological tests for *T. cruzi* and were selected at the Referral Outpatient Center for Chagas Disease at the Clinical Hospital of the *Universidade Federal de Minas Gerais* (UFMG). Patients were subjected to a standard screening protocol that included medical history, physical examination, ECG, laboratory and chest X-ray examinations, disease evolution by echocardiography and characterization according to the clinical classification of chronic Chagas disease [35]. None of the patients were undergoing etiological treatment nor had previously been treated for *T. cruzi* infection. Blood samples for BC (30 mL) and qPCR (5 mL) were collected at the same time for each patient. Amongst 91 patients, 44 subjects (48%) had two blood samples collected prospectively within a range interval of two and three years, aiming to evaluate the parasitemia over time in patients with chronic Chagas disease, with a total of 135 samples. This study comprises patients from a broad project on the clinical, parasitological, molecular and immunological studies that has been developed in our laboratory since 2011.

Ethical statement

The study was approved by the Research Ethic Committee of the *Universidade Federal de Minas Gerais* (protocol COEP-ETHIC 0559.0.203.000-11/2012/UFMG), and all participants provided written informed consent.

Blood culture

Blood culture (BC) was performed with 30 mL of venous blood collected in heparinized vacuum tubes and red cells were recovered from the plasma by centrifugation at 300 × g for 10 min at 4˚C [12]. The packed red blood cells were washed once and re-suspended in 6 mL of LIT (Liver Infusion Tryptose), mixed and distributed into six plastic tubes (Falcon, USA) containing 3 mL of LIT. The plasma supernatant was centrifuged at 900 × g for 20 min at 4˚C, and 5 mL LIT was added to the precipitated cells. All tubes were maintained at 28˚C, mixed gently twice a week, and examined monthly for up to 120 days. Microscopic examination was carried out in 10 μL aliquots of each preparation under a 22-mm² coverslip at a magnification of 400×.

Genotyping of *Trypanosoma cruzi* isolates

*T. cruzi* was isolated from all clinical samples with positive BC and the genotyping was performed by conventional PCR and RFLP, using three different parasite molecular targets: D7 domain 24Sα ribosomal (rRNA) gene [36], mitochondrial cytochrome oxidase subunit 2 gene (COII) [37], and the intergenic region of spliced leader genes [38], as markers for the six discrete typing units (DTUs) [39]. The following, TcI (Col1.7G2 Colombiana clone), TcII (JG), TcIII (222), TcIV (CAN III clone), TcV (3253 Lages-Silva et al.: unpublished data), and TcVI (CL) were used as reference strains and DTU controls [37,39].

DNA processing for absolute quantification by qPCR assays

For each patient, five milliliters of venous blood were collected and immediately mixed with an equal volume of 6M Guanidine Hydrochloride / 0.2 M ethylenediaminetetraacetic acid buffer (EDTA) solution, pH 8.0. The Guanidine-EDTA Blood lysates (GEB) were boiled during 15 min and stored at 4˚C, as previously described [40]. Extraction of DNA was processed
from 300 μL GEB using the High Pure PCR Template Preparation kit, according to the instruction provided by the manufacturer (Roche Diagnostics Corp., Indiana, USA). A linearized p-Zero plasmid containing a sequence of Arabidopsis thaliana was used as an exogenous internal reference (Internal Amplification Control, IAC) [23,25]. Each round of DNA extraction was performed using 12 blood samples, being 11 of patients and 1 of a negative control (GEB) for the DNA extraction. After extraction, DNA was stored at -20˚C until the time of use in qPCR.

Standard curves and positive controls

For the construction of the standard curve and the generation of positive controls used in qPCR, GEB from healthy individuals were spiked with \(10^6\) epimastigote forms/mL of T. cruzi, Y strain (spiked GEB\(^+\)). This strain corresponds to the discrete typing unit (DTU) II and was selected due to the high prevalence of this DTU and its association with human infection in the State of Minas Gerais/MG [16,41,42]. Total DNA was purified as previously described, followed by serial dilutions to obtain the concentrations of \(10^4\), \(10^3\), \(10^2\), \(10^1\), \(10^0\) and 0.5 par. eq./mL. As diluent, DNA extracted from blood sample of a healthy individual (GEB) was used. Each dilution was correlated to one point of the standard curve for the absolute quantification of parasite load in the clinical samples. DNA extracted from GEB\(^+\) spiked with T. cruzi to reach the concentrations of \(10^2\) and \(10^0\) par. eq./mL were also used as positive controls for the qPCR, in each assay.

Absolute quantification by qPCR assays

The qPCR was performed according to a methodology previously proposed [25], using the multiplex TaqMan system targeting the T. cruzi nuclear satellite DNA and IAC. The qPCR reactions were carried out with 5 μL of DNA, using FastStart Universal Probe Master Mix (Roche Diagnostics GmbH Corp., Mannheim, Germany) in a final volume of 20 μL. The amplifications were carried out in the Step One Plus Real-Time PCR system (Applied Biosystems, USA) using 750 nM of Cruzi 1 and Cruzi 2 primers, 50 nM of Cruzi 3 probe, 100 nM of IAC Fw and IAC Rv primers and 50 nM IAC Tq probe. The oligonucleotide sequences were: Cruzi 1 (ASTCGGCTGATCGTTTTCGA), Cruzi 2 (AATTCCTCCAAGCAGGATA) and Cruzi 3 probe (FAM-CACACACTGGACACCA-NFQ-MGB), IAC Fw (ACCGTCATGGAACAGCACGTA), IAC Rv (CTCCGGCAACACACCTATATAAT) and IAC Tq probe (VIC-AGCATCTGTTCTTGAGGT-NFQ-MGB) [25]. PCR cycling conditions were: 95˚C for 10 min, followed by 40 cycles at 95˚C 15s and 58˚C for 1 min. To analyze the results, the threshold was set at 0.02. Clinical samples were tested in duplicate, and considered positive when the fluorescent signal of both technical replicates crossed the threshold or negative when the fluorescent signal of both technical replicates did not cross the threshold.

Statistical analysis

Pearson’s correlation was used to verify the linear relationship between the parasite load of T. cruzi (par. eq./mL) detected in the clinical samples (qPCR), patient age and number of positive tubes in BC. Mann-Whitney-Wilcoxon and Kruskal-Wallis tests [43] were used, respectively, for comparison of T. cruzi parasite load (par. eq./mL) with the cardiac clinical form of patients and the different levels of heart disease. Pearson chi-squared test was used to compare the positivity of BC and qPCR in the clinical samples of patients with two blood collections (samples 1 and 2). Kappa coefficient concordance and 95% confidence intervals were used to quantify the degree of agreement between the results of BCs and qPCR [44,45] in clinical samples of patients with two blood collections. To confirm or refute the evidence found by the tests mentioned above, a 5% significance level was used.
Results

Characteristics of the study population

Overall, 25.3% (23/91) were patients with the chronic indeterminate form of Chagas disease and 74.7% (68/91) showed different degrees of cardiac involvement. Among the patients with the indeterminate form of Chagas disease, 34.8% (8/23) were male, with ages ranging from 33 to 70 years (mean of 44±10.3 years). Amongst patients with chronic Chagas cardiomyopathy, 66.2% (45/68) of were male, with ages ranging from 25 to 81 years (mean of 54±10.3 years).

Detection of *T. cruzi* by blood culture

Sixty-seven (49.6%) of the 135 clinical samples of patients with chronic Chagas disease presented positive BCs. Data concerning blood collection date, age, *T. cruzi* DTU, BC positivity, parasite load, clinical form of disease for each patient are given in Tables 1 and 2. A total of 63 *T. cruzi* isolates was obtained by positive blood culture. Of these, sixty-one are associate with discrete typing unit (DTU) II and two isolates from patients with cardiac and indeterminate form of the disease, respectively, were classified as DTU III or IV and DTU V or VI (Tables 1 and 2).

Among the 44 patients with two collected blood samples, 22.7% (10/44) showed positive BC in the two blood harvesting and 45.5% (20/44) showed negative BC in both samples. On the other hand, 15.9% (7/44) presented positive BC in the first and negative in the second sample. The same amount of samples 15.9% (7/44) presented negative BC in the first and positive in the second sample (Table 3). The analysis of BC results showed that the positivity observed in the first and second samples were the same [38.64% (17/44)], statistically demonstrating an equality in the first and second sample (p-value = 0.500) (Table 3).

*Trypanosoma cruzi* parasite load in chronic Chagas disease patients

Parasite loads were determined by qPCR absolute quantification in a TaqMan multiplex assay targeting *T. cruzi* satellite DNA and the internal control, IAC. It was possible to observe the dynamic range from $10^4$ to 0.5 parasite equivalents /mL, as previously reported [25,28], with efficiency of 89.5% and coefficient of linearity ($r^2$) of 0.99 (Fig 1).

Total qPCR positivity in clinical samples was 58.5% (79/135). Data on collection date, age, DTU, parasite loads and clinical form of the disease for each patient can be seen in Tables 1 and 2. The median parasite load of all positive samples was 1.18 par. eq./mL, varying between 0.01 and 116.10 par. eq./mL. The median parasite load of patients with indeterminate clinical form was 0.46 [0.24–3.02] par. eq./mL, varying between 0.01 and 85.81 par. eq./mL, and 1.74 [0.60–4.74] par. eq./mL for the cardiac patients ranging from 0.05 to 116.10 par. eq./mL (Tables 1 and 2). Analyzing the data from Figs 2 and 3, we found no correlation between *T. cruzi* loads and the age or clinical manifestation of the disease.

Clinical samples of 44 patients with two blood harvesting were evaluated and compared, and presented parasite loads with approximated values. Only clinical samples from patients 17, 18, 19, 24 and 66 showed differences in parasite load when the second sample was evaluated (Tables 1 and 2). The qPCR was positive in 31.8% (14/44) samples from patients with two blood harvesting and 22.7% (10/44) were negative in both samples. We observed that 9.1% (4/44) presented positive qPCR in the first and were negative in the second sample. In contrast, 36.4% (16/44) presented negative qPCR in the first and positive in the second sample (Table 3). The qPCR positivity increased from 40.9% (18/44) to 68.2% (30/44) (p = 0.005) with the inclusion of a second blood collection.
Association between qPCR assay and blood culture in chronic Chagas disease patients

Of the 135 screened samples, 38.5% (52/135) were tested positive for both qPCR and BC, 20.0% (27/135) were only positive for qPCR, 11.1% (15/135) were positive for BC but qPCR negative, and 30.4% (41/135) were negative for both assays (Table 4).

The parasitemia of Chagas disease patients was also evaluated by the number of positive tubes for BC and comparing with the parasite load obtained in qPCR of all positive clinical

Table 1. Comparison of blood culture, T. cruzi genotype and parasite load in asymptomatic patients with chronic Chagas disease.

| Blood Sample | Collection Date | Number of positive tubes | Blood culture | Parasite load ± SD (par. eq./mL) | DTU | Age |
|--------------|-----------------|--------------------------|---------------|----------------------------------|-----|-----|
| 009a         | 09/23/2011      | 1                        | POS           | 0.79±0.18                        | TcII | 58  |
| 0020a        | 10/21/2011      | 0                        | NEG           | 600                            | -   | 48  |
| 0020b        | 09/19/2014      | 2                        | POS           | 0.37±0.26                       | TcII | 33  |
| 0024a        | 10/25/2011      | 6                        | POS           | 36.82±3.50                      | TcII | 33  |
| 0024b        | 11/12/2014      | 5                        | POS           | 13.25±3.03                      | TcII | 33  |
| 0025a        | 01/11/2011      | 0                        | NEG           | 0.09±0.06                       | -   | 40  |
| 0040a        | 11/18/2011      | 1                        | POS           | 0.04±0.01                       | TcII | 62  |
| 0041a        | 11/18/2011      | 0                        | NEG           | 0.15±0.01                       | -   | 56  |
| 0041b        | 09/26/2014      | 0                        | NEG           | 0.09±0.06                       | -   | 36  |
| 0048a        | 11/29/2011      | 7                        | POS           | 85.84±6.22                      | TcII | 38  |
| 0052a        | 12/02/2011      | 1                        | POS           | 0.01±0.01                       | ND  | 37  |
| 0054a        | 12/06/2011      | 1                        | POS           | 0.30±0.16                       | TcV or VI | 38  |
| 0054b        | 11/14/2014      | 0                        | NEG           | 2.97±0.02                       | -   | 42  |
| 0061a        | 02/13/2012      | 0                        | NEG           | 0.32±0.08                       | -   | 42  |
| 0061b        | 10/03/2014      | 0                        | NEG           | 2.35±0.43                       | TcII | 70  |
| 0062a        | 10/03/2014      | 0                        | NEG           | 0.04±0.01                       | -   | 44  |
| 0062b        | 11/28/2014      | 2                        | POS           | 3.15±0.43                       | TcII | 70  |
| 0063a        | 03/02/2012      | 0                        | NEG           | 0.30±0.16                       | -   | 36  |
| 0063b        | 09/19/2014      | 0                        | NEG           | 2.97±0.02                       | 52  |
| 0064a        | 03/02/2012      | 1                        | POS           | 0.32±0.08                       | TcII | 70  |
| 0064b        | 11/28/2014      | 2                        | POS           | 3.15±0.43                       | TcII | 70  |
| 0067a        | 03/09/2012      | 2                        | POS           | 0.04±0.01                       | -   | 37  |
| 0069a        | 03/20/2012      | 0                        | NEG           | 0.30±0.16                       | -   | 37  |
| 0069b        | 10/03/2014      | 2                        | POS           | 0.01±0.01                       | TcII | 70  |
| 0072a        | 03/02/2012      | 0                        | NEG           | 0.30±0.16                       | -   | 37  |
| 0076a        | 04/03/2012      | 0                        | NEG           | 0.30±0.16                       | -   | 37  |
| 0078a        | 04/17/2012      | 0                        | NEG           | 0.30±0.16                       | -   | 37  |
| 0078b        | 09/12/2014      | 0                        | NEG           | 0.27±0.10                       | -   | 37  |
| 0084a        | 04/24/2012      | 0                        | NEG           | 0.55±0.15                       | -   | 37  |
| 0086a        | 04/24/2012      | 0                        | NEG           | 0.31±0.02                       | -   | 37  |
| 0086b        | 09/26/2014      | 2                        | POS           | 0.17±0.03                       | ND  | 37  |
| 0088a        | 04/05/2012      | 3                        | POS           | 0.96±0.34                       | TcII | 34  |
| 0092a        | 05/15/2012      | 0                        | NEG           | 0.09±0.06                       | -   | 34  |
| 0092b        | 11/18/2014      | 0                        | NEG           | 0.09±0.06                       | -   | 34  |
| 0094a        | 05/29/2012      | 0                        | NEG           | 0.31±0.02                       | -   | 34  |

SD: standard deviation, par. eq./mL: parasite equivalent per milliliter of blood, POS: positive, NEG: negative, ND: not done, a: first sample collected from the patient, b: second sample collected from the patient, DTU: discrete typing units.

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Table 2. Comparison of blood culture, *T. cruzi* genotype and parasite load in patients with different degrees of chronic Chagas disease cardiomyopathy.

| Blood sample | Collection date | CCC | Number of positive tubes | Blood Culture | Parasite load ± SD (par. eq/mL) | DTU | Age |
|--------------|-----------------|-----|--------------------------|---------------|---------------------------------|-----|-----|
| 001a         | 09/09/2011      | CCC5 | 7                         | POS           | 116.10 ±4.37                   | TcII| 59  |
| 002a         | 09/09/2011      | CCC3 | 5                         | POS           | 51.74 ±16.63                   | TcII| 41  |
| 003a         | 09/13/2011      | CCC4 | 2                         | POS           | 2.01±0.76                      | TcII| 56  |
| 004a         | 09/13/2011      | CCC3 | 1                         | POS           | 1.07±0.44                      | TcII| 51  |
| 005a         | 09/13/2011      | CCC5 | 5                         | POS           | 20.86±4.71                     | TcII| 71  |
| 006a         | 09/13/2011      | CCC3 | 0                         | NEG           | NEG                             | -   | 67  |
| 006b         | 09/26/2011      | CCC3 | 0                         | NEG           | 0.05±0.02                      | -   | -   |
| 007a         | 09/23/2011      | CCC5 | 1                         | POS           | 3.07±0.56                      | ND  | 45  |
| 008a         | 09/23/2011      | CCC5 | 3                         | POS           | 0.41±0.35                      | TcII| 55  |
| 0010a        | 09/27/2011      | CCC5 | 3                         | POS           | 4.71±0.06                      | TcII| 60  |
| 0012a        | 09/27/2011      | CCC4 | 0                         | NEG           | NEG                             | -   | 64  |
| 0012b        | 11/21/2014      | CCC4 | 0                         | NEG           | NEG                             | -   | -   |
| 0013a        | 10/04/2011      | CCC3 | 4                         | POS           | 0.47±0.27                      | TcII| 48  |
| 0014a        | 10/04/2011      | CCC3 | 0                         | NEG           | NEG                             | -   | 81  |
| 0015a        | 10/04/2011      | CCC3 | 0                         | NEG           | 0.41±0.07                      | -   | 34  |
| 0015b        | 11/21/2014      | CCC3 | 0                         | NEG           | 2.65±1.88                      | -   | -   |
| 0016a        | 10/21/2011      | CCC4 | 1                         | POS           | 0.29±0.18                      | TcII| 46  |
| 0016b        | 09/19/2014      | CCC4 | 0                         | NEG           | 0.06±0.02                      | -   | -   |
| 0017a        | 10/21/2011      | CCC1 | 5                         | POS           | 24.51±3.23                     | TcII| 58  |
| 0017b        | 11/12/2014      | CCC1 | 4                         | POS           | 3.74±2.78                      | TcII| 70  |
| 0018a        | 10/21/2011      | CCC2 | 5                         | POS           | 12.79±1.49                     | TcII| 46  |
| 0018b        | 09/12/2014      | CCC2 | 1                         | POS           | 0.75±0.54                      | TcII| 58  |
| 0019a        | 10/21/2011      | CCC3 | 5                         | POS           | 36.09±4.53                     | TcII| 58  |
| 0019b        | 11/14/2014      | CCC3 | 7                         | POS           | 5.34±2.22                      | TcII| 50  |
| 0021a        | 10/25/2011      | CCC2 | 2                         | POS           | 0.41±0.35                      | TcII| 50  |
| 0022a        | 10/25/2011      | CCC3 | 1                         | POS           | 14.93±2.86                     | ND  | 58  |
| 0023a        | 10/25/2011      | CCC5 | 5                         | POS           | 2.52±0.022                     | TcII| 70  |
| 0023b        | 11/14/2014      | CCC5 | 1                         | POS           | NEG                             | TcII| 58  |
| 0026a        | 11/01/2011      | CCC1 | 4                         | POS           | 0.69±0.20                      | TcII| 25  |
| 0027a        | 11/01/2011      | CCC5 | 0                         | NEG           | NEG                             | -   | 56  |
| 0027b        | 08/29/2014      | CCC5 | 0                         | NEG           | NEG                             | -   | -   |
| 0028a        | 11/04/2011      | CCC5 | 0                         | NEG           | NEG                             | -   | 57  |
| 0029a        | 11/04/2011      | CCC5 | 0                         | NEG           | NEG                             | -   | 59  |
| 0030a        | 11/04/2011      | CCC5 | 0                         | NEG           | NEG                             | -   | 52  |
| 0031a        | 11/04/2011      | CCC5 | 2                         | POS           | 4.75±0.11                      | TcII| 59  |
| 0032a        | 11/07/2011      | CCC3 | 4                         | POS           | 9.39±6.24                      | TcII| 49  |
| 0033a        | 11/07/2011      | CCC1 | 0                         | NEG           | NEG                             | -   | 67  |
| 0033b        | 09/26/2014      | CCC1 | 0                         | NEG           | 0.39±0.25                      | -   | -   |
| 0034a        | 11/07/2011      | CCC5 | 5                         | POS           | 2.79±0.79                      | TcII| 48  |
| 0035a        | 11/07/2011      | CCC5 | 1                         | POS           | NEG                             | TcII| 68  |
| 0036a        | 11/07/2011      | CCC3 | 0                         | NEG           | NEG                             | -   | 77  |
| 0037a        | 11/07/2011      | CCC4 | 4                         | POS           | 1.47±0.02                      | TcII| 38  |
| 0038a        | 11/07/2011      | CCC5 | 5                         | POS           | 1.46±0.76                      | TcII| 52  |
| 0038b        | 12/05/2014      | CCC5 | 0                         | NEG           | 2.06±1.07                      | -   | -   |
| 0039a        | 11/18/2011      | CCC3 | 0                         | NEG           | NEG                             | -   | 44  |
| 0039b        | 11/12/2014      | CCC3 | 0                         | NEG           | NEG                             | -   | -   |
| 0042a        | 11/18/2011      | CCC5 | 1                         | POS           | NEG                             | TcII| 55  |

(Continued)
| Blood sample | Collection date | CCC | Number of positive tubes | Blood Culture | Parasite load ± SD (par. eq./mL) | DTU | Age |
|--------------|----------------|-----|--------------------------|---------------|----------------------------------|-----|-----|
| 0043a        | 11/22/2011     | CCC5 | 0                        | NEG           | NEG                              | -   | 58  |
| 0043b        | 09/26/2014     | CCC5 | 0                        | NEG           | 0.07±0.01                        | -   |     |
| 0044a        | 11/22/2011     | CCC5 | 2                        | POS           | 0.91±0.38                        | TcII| 66  |
| 0044b        | 11/14/2014     | CCC5 | 2                        | POS           | 0.72±0.51                        | TcII|     |
| 0046a        | 11/29/2011     | CCC5 | 4                        | POS           | 1.86±0.63                        | TcII| 61  |
| 0047a        | 11/29/2011     | CCC3 | 2                        | POS           | NEG                              | TcII| 60  |
| 0049a        | 12/02/2011     | CCC5 | 2                        | POS           | 1.18±0.23                        | TcII| 59  |
| 0049b        | 12/05/2014     | CCC5 | 0                        | NEG           |                                  |     |     |
| 0050a        | 12/02/2011     | CCC5 | 1                        | POS           | 1.71±1.02                        | TcII or IV| 58  |
| 0050b        | 11/14/2014     | CCC5 | 0                        | NEG           |                                  | -   |     |
| 0053a        | 12/06/2011     | CCC5 | 0                        | NEG           | NEG                              | -   | 55  |
| 0053b        | 11/18/2014     | CCC5 | 1                        | POS           | NEG                              | TcII|     |
| 0055a        | 12/06/2011     | CCC5 | 0                        | NEG           |                                  | -   | 69  |
| 0055b        | 10/03/2014     | CCC5 | 0                        | NEG           | 0.94±0.17                        | -   |     |
| 0056a        | 12/06/2011     | CCC5 | 2                        | POS           | 4.98±2.12                        | TcII| 38  |
| 0056b        | 11/28/2014     | CCC5 | 0                        | NEG           | 1.17±0.52                        | -   |     |
| 0057a        | 12/06/2011     | CCC5 | 2                        | POS           | 3.57±2.99                        | TcII| 36  |
| 0058a        | 02/10/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 58  |
| 0059a        | 02/10/2012     | CCC4 | 1                        | POS           | NEG                              | TcII| 41  |
| 0059b        | 11/28/2014     | CCC4 | 1                        | POS           | NEG                              | TcII|     |
| 0060a        | 02/10/2012     | CCC5 | 0                        | NEG           | 33.30±0.05                       | -   | 53  |
| 0065a        | 03/06/2012     | CCC4 | 5                        | POS           | NEG                              | TcII| 53  |
| 0066a        | 03/06/2012     | CCC5 | 0                        | NEG           | 17.31±1.82                       | -   | 57  |
| 0066b        | 29/08/2014     | CCC5 | 0                        | NEG           | 0.22±0.07                        | -   |     |
| 0068a        | 03/20/2012     | CCC5 | 2                        | POS           | NEG                              | TcII| 67  |
| 0068b        | 12/05/2014     | CCC5 | 4                        | POS           | 22.33±0.01                       | TcII|     |
| 0070a        | 03/23/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 56  |
| 0070b        | 10/03/2014     | CCC5 | 0                        | NEG           | 0.24±0.17                        | -   |     |
| 0071a        | 03/27/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 44  |
| 0071b        | 11/14/2014     | CCC5 | 0                        | NEG           | NEG                              | -   |     |
| 0073a        | 03/27/2012     | CCC5 | 2                        | POS           | 13.89±4.46                       | TcII| 54  |
| 0074a        | 03/27/2012     | CCC5 | 1                        | POS           | 2.04±1.11                        | TcII| 54  |
| 0075a        | 03/27/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 56  |
| 0075b        | 09/26/2014     | CCC5 | 0                        | NEG           | 0.24±0.06                        | -   |     |
| 0077a        | 04/03/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 59  |
| 0079a        | 04/17/2012     | CCC3 | 0                        | NEG           | NEG                              | -   | 53  |
| 0079b        | 09/12/2014     | CCC3 | 1                        | POS           | 0.68±0.61                        | TcII| 53  |
| 0080a        | 04/17/2012     | CCC2 | 1                        | POS           | 1.98±1.01                        | TcII| 55  |
| 0081a        | 04/20/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 66  |
| 0081b        | 08/29/2014     | CCC5 | 0                        | NEG           | NEG                              | -   |     |
| 0083a        | 04/20/2012     | CCC3 | 0                        | NEG           | NEG                              | -   | 35  |
| 0083b        | 11/21/2014     | CCC3 | 2                        | POS           | 2.59±0.98                        | TcII|     |
| 0087a        | 04/24/2012     | CCC5 | 1                        | POS           | 0.78±0.56                        | TcII| 35  |
| 0089a        | 05/08/2012     | CCC1 | 4                        | POS           | 3.65±1.18                        | TcII| 53  |
| 0089b        | 09/12/2014     | CCC1 | 2                        | POS           | NEG                              | TcII|     |
| 0090a        | 05/15/2012     | CCC5 | 0                        | NEG           | NEG                              | -   | 54  |
| 0090b        | 09/12/2014     | CCC5 | 1                        | POS           | 1.74±0.36                        | TcII|     |

(Continued)
samples. Fig 4 shows a significant correlation between the number of positive tubes in BC and the parasite load of T. cruzi in clinical samples (p-value < 0.0001). We also observed significant correlation between the number of BC positive tubes and the parasite load in the individual analysis of the first and second samples in patients with two blood harvesting (p-value < 0.0001) (Fig 4A and 4B).

**Discussion**

Due to the sub-patent and transient parasitemia, the direct detection of T. cruzi in the chronic phase of Chagas disease requires biological amplification methods such as blood culture and xenodiagnosis. These methods are more complex, expensive, time-consuming and require special biosecurity conditions in the laboratory [46,47]. Previous reports have shown that multiplex real-time qPCR assay allowed detection and quantification of parasite DNA from clinical samples with variable levels of reliability, complexity, selectivity and analytical sensitivity [21,23–25,28,31,34], also permitting the T. cruzi genotyping in clinical samples [33, 48].

In this study, blood samples from chronic Chagas disease patients with well-defined clinical forms were evaluated, using blood culture (BC) and multiplex quantitative real-time PCR (qPCR) to the detection and quantification of T. cruzi DNA in human blood. All patients presented positive conventional serology for T. cruzi and had not received any specific etiological treatment. For 44 patients, two blood samples were collected, in an interval of two to three years, in order to evaluate the parasite load of chronic patients in the period of 2011–2014. Herein, BC was positive in 49.6% (67/135) of the clinical samples. In a previous study from our group, we detected 54.9% (50/91) of positive BCs, corresponding to first samples collected.

| Blood sample | Collection date | CCC | Number of positive tubes | Blood Culture | Parasite load ± SD (par. eq./mL) | DTU       | Age |
|--------------|----------------|-----|--------------------------|---------------|---------------------------------|-----------|-----|
| 0091a        | 05/15/2012     | CCC3 | 0                        | NEG           | NEG                             | -         | 59  |
| 0091b        | 11/18/2014     | CCC3 | 0                        | NEG           | NEG                             | -         |     |
| 0093a        | 05/15/2012     | CCC5 | 1                        | POS           | 0.99±0.27                       | TcII      | 58  |
| 0093b        | 09/05/2014     | CCC5 | 0                        | NEG           | 0.07±0.04                       | -         |     |
| 0095a        | 05/29/2012     | CCC4 | 3                        | POS           | 0.51±0.38                       | TcII      | 46  |
| 0096a        | 05/29/2012     | CCC2 | 0                        | NEG           | 0.09±0.02                       | -         | 49  |

CCC1 to 5: chronic Chagas cardiomyopathy in different degrees of cardiac involvement, SD: standard deviation, par. eq./mL: parasite equivalent per milliliter of blood, POS: positive, NEG: negative, ND: not done, a: first sample of the patient, b: second sample of the patient, DTU: discrete typing units.

Table 3. Percentage of concordance, point and interval estimates of kappa coefficient according to qPCR and blood culture methods in 44 Chagas disease patients with two blood harvesting.

| Method         | 1st sample | 2nd sample | Percentage(Number of patients/total) | Agreement Coefficient | Type of agreement |
|----------------|------------|------------|--------------------------------------|-----------------------|-------------------|
| qPCR           | Positive   | Positive   | 31.8 (14/44)                         | 0.083 [-0.212; 0.379] | Slight            |
|                | Positive   | Negative   | 9.1 (4/44)                           |                       |                   |
|                | Negative   | Positive   | 36.4 (16/44)                         |                       |                   |
|                | Negative   | Negative   | 22.7 (10/44)                         |                       |                   |
| Blood Culture  | Positive   | Positive   | 22.7 (10/44)                         | 0.329 [0.034; 0.624]  | Fair              |
|                | Positive   | Negative   | 15.9 (7/44)                          |                       |                   |
|                | Negative   | Positive   | 15.9 (7/44)                          |                       |                   |
|                | Negative   | Negative   | 45.5 (20/44)                         |                       |                   |

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from the 91 patients [49]. The vast majority of data in the literature have reported BC positivity ranging from 40 to 70% [6,15,41,49–52]. In the patients with two blood samples, blood culture positivity rate was the same (38.6%) for the first and second blood samples. However, the degree of agreement between the two samples was fair, indicating that a patient with positive BC in the first sample can present positive or negative BC in the analysis of the second blood sample. To the patient 0054, for example, it was observed a positive blood culture at the first

Fig 1. Dynamic range for Trypanosoma cruzi quantification by Real Time qPCR. TaqMan qPCR was carried out with serial diluted DNA extracted from blood spiked with T. cruzi [Y strain], ranging from $10^4$ to 0.5 par. eq./mL (parasite equivalent per milliliter of blood).

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Fig 2. Relationship between parasite load and age of patients with chronic Chagas disease. The number of positive qPCR results for the first and second clinical samples was respectively, 49 (A) and 30 (B). LOQ: Limit of Quantification [25].

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Our results confirm previous findings and indicate that at least two blood samples should be collected from chronic Chagas disease patients in order to detect circulating *T. cruzi* [15, 16, 41, 53].

The qPCR method has shown higher potential to diagnose and estimate the parasite load, despite the subpatent and transient parasitemia that occurs in the chronic phase of Chagas disease. According to this methodology, the Limit of quantification was reported as 1.53 parasite equivalents/mL [25], which means that samples with parasite load below this limit can be considered detectable but not quantifiable. Nevertheless, as the majority of samples of patients from Brazil are in this condition, we decided to report the parasite load for all the positive samples. Thus, *T. cruzi* DNA was detected in 58.5% (79/135) of blood samples by qPCR and the median parasite load was 1.18 [0.39–4.23] par. eq./mL, varying between 0.01 and 116.10 par. eq./mL. On the other hand, *T. cruzi* k-DNA was detected by conventional PCR in 98.9% (90/91) of the first samples collected from these patients [49], demonstrating more efficiency in detecting the parasite in the peripheral blood of infected patients when compared to BC and qPCR. However, conventional PCR does not allow monitoring parasite load in peripheral blood of chronic Chagas disease patients and as a criteria for the isolation of *T. cruzi*, emphasizing the importance of BC and qPCR for new biological, molecular, biochemical, immunological, genetic studies of parasitic populations and parasite load monitoring.

Our findings corroborate with other studies using qPCR to infer parasite load from blood of Brazilian, Argentines, Bolivians, Colombians and Mexicans chronic Chagas disease patients, where the median parasite load ranged from 1.23 to 4.0 par. eq./mL [25, 27, 28, 31, 34].

Table 4. Percentage of agreement, point and interval estimates of kappa coefficient of 135 blood samples obtained from 91 Chagas disease patients from different endemic regions of the state of Minas Gerais (southern Brazil).

| qPCR  | Blood culture | Percentage (Number of patients/total) | Agreement Coefficient | Type of agreement |
|-------|---------------|---------------------------------------|-----------------------|-------------------|
| Positive | Positive | 38.5 (52/135) | 0.374 [0.205; 0.542] | Fair |
| Negative | Positive | 11.1 (15/135) | | |
| Positive | Negative | 20.0 (27/135) | | |
| Negative | Negative | 30.4 (41/135) | | |

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78 par. eq./mL [33], higher than previously described for the same group of patients, fluctuating between <0.1 and 7.9 par. eq./mL [30]. Studies have shown that qPCR has been used for the detection and quantification of T. cruzi load in blood, serum, heart tissue, cord blood, fecal samples of Triatoma infestans (xenodiagnosis), skin tissue samples from Chagas disease patients in the acute and chronic phases and also to differentiate the parasite DTUs [21–34,48], suggesting that genetic differences between parasite strains can influence the parasitic load and PCR positivity [20,22,26,27].

In this study, the detection rate for T. cruzi by qPCR increased from 40.9% to 68.2% in patients with two blood samples collected at different time points; however, the concordance analysis indicated a slight correlation between the samples, with qPCR results from the first sample not often matching the results observed in the second sample.

We performed a comparative analysis of qPCR positivity and blood culture. The two techniques were positive in 38.5% of the samples. Discordant results were observed in 31.1% of the samples, being 11.1% of them positive by BC and qPCR negative, while in 20.0% only the qPCR gave positive results. In contrast, 30.4% of the samples were negative by both techniques. This finding confirms the occurrence of intermittent parasite levels and depends on the number of circulating parasite at the time of blood collection and the number of samples analyzed from the same patient, since in the life cycle of T. cruzi, the release of trypomastigote forms does not occur in a synchronized way. So, the presence of the parasite in peripheral blood at a given time depends on the parasite’s biological cycle, as well as on the immunological equilibrium among parasite and host [54]. Differences in positivity between qPCR and BC can be explained by low parasitemia, probably below the detection limit of the two techniques.

Understanding the structure of T. cruzi population is essential due to the links between parasite transmission cycles and the infection/disease. T. cruzi isolates were analyzed by rDNA, COII and SL-IR molecular markers aimed at detecting the six DTUs of T. cruzi. Most isolates from the patients were associated with DTU II. Two isolates from patients with cardiac and indeterminate clinical form, respectively, were also identified associated with DTU III or IV and DTU V or VI [49]. These data were consistent with previous studies showing that DTU II was associated with human infection in the state of Minas Gerais, Brazil [16,42,55].

Consistent with previous studies, we did not find a correlation between neither T. cruzi parasite load nor age and clinical presentation of Chagas disease [21,24,26,27,31,33]. This lack of correlation was also observed in another Brazilian cohort comprising 40 patients with chronic
Chagas disease [27]. In our recent study, we did not observe significant difference between BC results, age of patients and clinical form [49]. We believe that the lack of association between T. cruzi parasite load and forms of the disease might be related to parasite tropism for specific organs or host tissues. After their penetration into human tissues, some T. cruzi populations could disappear, while others could invade different tissues, which would be responsible for the various clinical manifestations in Chagas disease. Thus, parasite obtained in the peripheral blood may not represent the populations of T. cruzi present in other tissues and/or organs of the patients. Furthermore, it is more important to analyze parasite present in the bloodstream at different time periods, increasing the chance of recovery of different T. cruzi subpopulations and making possible the analysis of its importance in the pathogenesis of Chagas disease [56,57].

Finally, our results showed a positive correlation between T. cruzi parasite load estimated by qPCR and number of positive BC tubes, demonstrating a high potential of qPCR for diagnosis and monitoring parasite load in peripheral blood of chronic Chagas disease patients. In another work, the parasitic loads of 15 GEB samples from Brazilian chagasic patients were compared with hemoculture. Despite the small number of samples, these authors demonstrated a good correlation between the parasitic load of T. cruzi detected by qPCR and the positivity of blood culture. [28].

Our results suggest that qPCR has diagnostic advantages for T. cruzi detection compared to BC, as it requires low blood volume and shorter processing time, allowing analysis of several samples at the same time. In addition, this tool presents high sensitivity for T. cruzi detection and quantification with lower risk of sample contamination when compared to BC. Another advantage in the use of the multiplex TaqMan assay is the possibility of checking the quality of patients’ blood processing and DNA extraction, especially to avoid false negative results [21,24–27,31,34]. On the other hand, BC has been frequently used for the isolation of T. cruzi, a necessary procedure for studies on biological, biochemical, immunological and some genetic aspects of parasite populations. Thus, BC is the most efficient technique for T. cruzi isolation and its amplification using LIT culture medium [15,16,41].

Taken together, our data suggest that qPCR can be an auxiliary tool for studies that require the isolation of T. cruzi parasite from the bloodstream of chronic Chagas disease patients, after establishing a cut-off for parasite load assuring a relative success rate for their isolation using blood culture technique.

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