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Validation of quantitative real-time PCR for the *in vitro* assessment of antileishmanial drug activity

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ABSTRACT

In vitro assays play an important role in the discovery and development of new antileishmanial drugs. The classic macrophage-amastigote models using murine peritoneal macrophages or human-monocyte derived macrophages as host cells are useful for drug screening. A major limitation of these models is the dependence on microscopic counting, a time-consuming and subjective method of analysis. The present study describes a detailed protocol for applying quantitative real-time PCR (qPCR) as an accurate and sensitive tool to assess parasite load in an amastigote-macrophage model. This assay can be performed in a standardized medium-to-high throughput procedure, replacing traditional readout of number of amastigote per macrophages by DNA load measurement.

1. Introduction

Leishmaniasis is one of the world's most neglected diseases. About 12 million people are currently infected, and an estimated 2 million new cases (1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis) occur annually (World Health Organization, 2011). Chemotherapy and vector control are available tools in combating this disease, as effective vaccines are still under development (Chawla and Madhubala, 2010).

Pentavalent antimonials have been used in the treatment of leishmaniasis for more than 60 years, despite their disadvantages that includes parenteral administration, long-term therapy, and potentially severe side effects associated with dosage and toxicity. In addition, acquired resistance to pentavalent antimonials has been developed in the high-prevalence, high-transmission epidemic areas of India (Seifert, 2011).

Amphotericin B is currently used as first line drug in areas with high rates of unresponsiveness to antimonials and as second line treatment elsewhere. Miltefosine and paromomycin have recently been used, but all these options suffer from limitations such as cost, specific toxicities and parenteral administration (Croft et al., 2006; de Oliveira-Silva et al., 2008; Dujardin et al., 2010; Seifert, 2011). Thus, the development of new antileishmanial chemotherapy is urgently needed to efficiently combat and eliminate this disease.

In vitro assays play an important role in primary screening for new antileishmanial drugs. Macrophage-amastigote model using murine peritoneal macrophages or human monocyte-derived macrophages is the most widely used assay for testing drugs against *Leishmania* species (Gupta and Nishi, 2011). This model yield essential information on drug activity in the parasite's natural environment and identify species/strain variations in drug sensitivity. The activity of the test drug is usually measured by microscopic counting of the percentage of infected cells or the number of amastigote per cell through of 50–300 macrophages examination. This is a time-consuming and subjective method of analysis, not adaptable to medium-to-high throughput screening models (Fumarola et al., 2004; Croft et al., 2006; Sereno et al., 2007; Gupta and Nishi, 2011).

In this study, quantitative real-time PCR (qPCR) was validated as a reliable tool to assess parasite load in amastigote-macrophage assays. Measuring amphotericin B activity against *Leishmania. (Leishmania) infantum* (syn. *L (L.) chagasi*), qPCR analysis compared favorably to the classic method of microscopic counting of intracellular amastigotes.

2. Material and methods

2.1. Antibiotics

Amphotericin B (Anforicin B; Cristália, Brazil) was obtained as a sterile powder. The powder was reconstituted with sterile water

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and diluted to the appropriate concentration in RPMI-1640 medium (Sigma–Aldrich, St. Louis, USA) for amastigote-macrophage cultures immediately before assays were performed.

2.2. Parasites and cultivation of amastigote-like forms

L. infantum strain MHOM/BR/70/BH46 was used in this study. Amastigote-like forms were transformed from stationary-phase promastigotes, cultivated in Schneider's insect medium (Sigma–Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA) at pH 7.2, 26 °C. Promastigotes were centrifuged (10 min/1000 g), resuspended in Schneider's medium, and transformed according to the species-specific protocol for *L. infantum* at 32 °C, pH 7.2 in 20% FBS for 6 days.

2.3. Amastigote-macrophage assay for L. infantum

Balb/c mice were injected intraperitoneally with 1.5 mL of 3% thioglycolate medium (Biobrás, Montes Claros, Brazil). After 96 h, peritoneal macrophages were harvested by peritoneal lavage using cold RPMI-1640 medium (Sigma–Aldrich). Cells were counted, centrifuged, and resuspended at a concentration of 4 10^5/mL in RPMI medium without supplements. Sterile round glass coverslips (13 mm) were placed in each well of 24-well culture plates. Five hundred microliters of the macrophage suspension was added to each well, and the macrophages were allowed to attach to the coverslips for 2 h at 37 °C in 5% CO_2. After 2 h, the medium was removed from the wells and replaced with 500 μL of warm (37 °C) RPMI containing 10% FBS plus penicillin (50 U/mL) and streptomycin (50 μg/mL). A suspension of 3.6 10^6/mL amastigote-like *L. infantum* was added to each well in 500 μL of RPMI (macrophage:parasite ratio of 1:9), after 18–24 h. The plates were incubated for 4 h at 37 °C in 5% CO_2, and the medium was aspirated to remove free-floating parasites. Fresh RPMI (1 mL) containing amphotericin B or not at the appropriate concentrations (0.3, 0.12, 0.048, 0.0196 and 0.0078 μg/mL) was added to the wells in triplicate. The plates were incubated for 72 h at 37 °C in 5% CO_2.

Three independent experiments were performed in triplicate for each drug concentration to determine amphotericin B activity in the amastigote-macrophage model using microscopic counting. Two of these independent experiments were also assessed by qPCR.

Animals were handled according to local and federal regulations, and the research protocols were approved by the Fiocruz Committee on Animal Research (protocol P-0321/06; license L-0024/8).

2.4. Drug activity measured by microscopic counting of the amastigote-macrophage assay

The medium was aspirated, and the coverslips were removed, methanol fixed, air-dried, and glued to microscope slides. After staining with Giemsa, 100 cells were counted along the border of the coverslips under optical microscope with a 100x oil-immersion objective. The results were expressed as the number of amastigotes-100 macrophages in treated and untreated cultures.

2.5. Drug activity measured by qPCR analysis of the amastigote-macrophage assay

The medium was aspirated, and the coverslips were removed. A simple protocol using Trypsin/EDTA was developed to detach the macrophages from the coverslips. In new 24-well culture plates, 150 μL of 0.25% trypsin/EDTA (Invitrogen, Camarillo, CA, USA) was applied to the coverslips and removed immediately. The plates containing the treated coverslips were incubated for 5 min at 37 °C in 5% CO_2. Each coverslip was washed five times with 400 μL of sterile PBS (phosphate buffered saline), and the entire volume (1200 μL) was collected in a Falcon tube. The coverslips were placed in the tubes, which were then centrifuged at 300 g for 7 min. Only 400 μL of the supernatant was left for the cell pellets, and cell concentrations were determined using a Neubauer chamber. The tubes were stored at 70 °C until the DNA extraction.

Total DNA was extracted using the QIAamp® DNA Mini kit (Qiagen, GmbH, Hilden, Germany) following the protocol for cultured cells, increasing the volumes of Buffer AL, proteinase K and ethanol (96–100%) accordingly for a 400 μL sample volume. Additionally, 5 μg of poly dA carrier DNA (Sigma–Aldrich) was added to each sample. The final elution volume was 60 μL. The concentration and purity of the extracted DNA were determined spectrophotometrically by reading A_{260} and A_{280} on a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

Two independent assays for the detection and quantification of *Leishmania* and mouse DNA were developed using the Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

For the *Leishmania* assay, the target DNA was the small-subunit ribosomal RNA (SSU rRNA) gene, which is conserved among all *Leishmania* species. About 160 copies of the SSU rRNA gene are present in every *Leishmania* cell (van Eys et al., 1992). The *Leishmania* assay consisted of the primers LEIS.U1 (5⁰-AAGTGC TTTCCCATCGCAACT-3⁰) and LEIS.L1 (5⁰-GACGCACTAAACCC CCTCCAA-3⁰), designed to amplify a 67-bp fragment and the fluorogenic probe LEIS.P1 (FAM 5⁰-CGGTTCGGTGTGTGGCGCC-3⁰TAMRA). These primers were chosen to confer sensitivity and specificity satisfactory to the test (Wortmann et al., 2001). Each sample amplification was performed in triplicate in a final volume of 20 μL, including 3 μL of template DNA, 10 μL of TaqMan® Universal PCR Master Mix 2 (Applied Biosystems), 0.3 μM of each primer and 0.25 μM of the probe. The optimized PCR amplification conditions were 50 °C for 2 min (UNG activation), 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The threshold of detection and the baseline were automatically determined using StepOne™ Software v2.1. A blank consisting of the reaction mixture and water instead of DNA template was included in each qPCR run.

For the mouse assay, the ACTB reference gene was used as target (Giulietti et al., 2001). The primers 5⁰-AGAGGGAAATCGTGCGT-GAC-3⁰ (forward) and 5⁰-CAATAGTGATGACCTGGCCGT-3⁰ (reverse) were designed to amplify a 138-bp fragment. Reaction mixtures contained 12.5 μL of Syber® Green PCR Master Mix 2X (Applied Biosystems), 0.1 μM of each primer, and 3 μL of DNA template in a final volume of 25 μL. Thermal cycling comprised an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To confirm specificity of the mouse assay, a final melting curve analysis was conducted with incubation at 95 °C for 15 s, 60 °C for 1 min, followed by a continuous temperature increase from 60 to 95 °C at a rate of 0.3 °C/s. The melting temperature of the amplicons was automatically determined by software analysis. Each sample amplification was performed in triplicate, and a blank consisting of the reaction mixture and water instead of DNA template was included in each qPCR run.

Standard curves were prepared for each assay using known quantities of pCR® 4 TOPO® vector (Invitrogen, São Paulo, Brazil) containing the cloned mouse gene (ACTB; 138 bp) and the 67 bp *L. infantum* SSU rRNA fragment. The recombinant plasmids were serially diluted 1:10 to create each standard curve. For the *Leishmania* assay, 160 copies of the SSU rRNA gene represented a single parasite cell. The quality parameters of the standard curves, including PCR efficiency, linear dynamic range and correlation coefficient, were obtained by software analysis.

Parasite load was quantified by two distinct methods. In the first method, the total DNA concentration of each sample was

divided by the highest value of all samples, resulting in a DNA normalization factor (F). Parasite density (PD) was calculated using the equation proposed by Rolão et al. (2004):

PD = [(*N* *P*)/*F*] [100 macrophages/number of macrophages, as quantified using a Neubauer chamber]. PD refers to parasite density (number of amastigotes-100 macrophages), *N* is the number of parasites obtained and *P* is the proportion between the amount of DNA used in the technique (3 μL) and the total volume of the eluted DNA (60 μL). In the second method, the parasite load was expressed by the *Leishmania* DNA load (relative copy number of the 67 bp SSU rRNA fragment) normalized against the reference gene ACTB, according to Overbergh et al. (1999). ACTB copy numbers for the target samples were divided by the highest ACTB value obtained in the experiment, resulting in a correction factor used for normalization.

Results were expressed as the number of amastigotes-100 macrophages (or the *Leishmania* DNA load) in treated and untreated cultures.

2.6. Data analysis

Data were processed with GraphPad Prism version 5.00 for Windows (San Diego, CA, USA). All quantitative variables were individually assessed with the one-sample Kolmogorov–Smirnov test for normality. The parasite load measured by microscopic counting and both methods of qPCR assessment were analyzed using the Pearson's parametric correlation coefficient. In order to determine the variability of the qPCR assay, intra-assay (repeatability) and inter-assay (reproducibility) precision levels were measured by comparing the means ± the S.D. and reported as coefficients of variation (CVs, S.D./mean 100%). The 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively) of amphotericin B were calculated using sigmoid dose–response curves and the 95% confidence intervals (95% CI) were included. The level of significance was set at $P < 0.05$.

3. Results

3.1. qPCR assay performance

In the initial experiments, the performance of the *Leishmania* assay was evaluated by making serial dilutions ranging from 2.54 10^2 to 2.54 10^8 copies of the linearized plasmid DNA containing the 67-bp fragment (equivalent to 1.5–1.5 10^6 *Leishmania* cells). The mean standard curve calculated from five independent experiments was linear over an at least 6-$\log_{10}$ range of DNA concentrations, with a correlation coefficient of 0.99. The PCR efficiency of amplification was 99.9%, and the inter-assay coefficients of variance, calculated from triplicates of the same 10-fold DNA dilutions, ranging from 1.5 10^6 to 15 parasites and performed on separated runs were 5.17%, 6.39%, 5.17%, 4.90%, 4.98% and 4.89%, respectively. Intra-assay coefficients of variance, calculated from triplicates of the same six different concentrations and performed on the same plate were 0.16%, 0.73%, 0.50%, 0.43%, 1.98% and 2.06%, respectively. Similar results were obtained with the mouse assay (data not shown).

The *Leishmania* assay was positive for samples with 2.54 10^2 copies of linearized plasmid DNA, corresponding to 1.5 *Leishmania* cells. The limit of detection was also evaluated by making serial dilutions of *L. infantum* genomic DNA in water to obtain the points of the curve spanning from 12,000 to 0.012 pg/μL. To convert the amount of DNA detected into parasite numbers, the genome size of a diploid *L. infantum* cell (assumed to be 3.2 10^7 bp – http://www.sanger.ac.uk) was converted into a mass equivalent, yielding a value of approximately 0.070 pg. As the assay uses 3 μL of DNA template, 0.036 pg of genomic DNA was detected, representing less than a single parasite cell.

3.2. Activity of amphotericin B on L. infantum in the amastigote-macrophage assay

The IC_{50} and IC_{90} values for amphotericin B activity against intracellular *L. infantum* amastigotes are shown in Table 1. These values were determined using sigmoid dose–response curves from three independent microscopic counting experiments and two independent qPCR experiments using the amastigote-macrophage model. The activity of amphotericin B was determined at concentrations of 0.3, 0.12, 0.048, 0.0196 and 0.0078 μg/mL; an IC_{50} of 0.02 μg/mL (95% CI: 0.01–0.03) and an IC_{90} of 0.04 μg/mL (95% CI: 0.01–0.15) were observed when assessed by microscopic counting. These same results were obtained when assessed by qPCR regardless of the method of parasite load quantification (number of amastigotes-100 macrophages or *Leishmania* DNA load).

3.3. Correlation between parasite load estimated by microscopic counting and qPCR in a L. infantum amastigote-macrophage model

The correlation between the mean parasite load in a *L. infantum* amastigote-macrophage model as estimated by microscopic counting and both methods of qPCR assessment are shown in Fig. 1. A Pearson's correlation coefficient of 0.987 ($P = 0.001$) was found when the parasite load was determined by microscopic counting or by qPCR, with the results expressed as the number of amastigotes-100 macrophages (Fig. 1A). When the correspondence between microscopic counting and qPCR (expressed as *Leishmania* DNA load) was assessed, the observed correlation coefficient was 0.995 ($P < 0.001$) (Fig. 1B).

4. Discussion

Real-time quantitative PCR (qPCR) is considered a very promising tool for the detection and quantification of *Leishmania* in different human clinical samples, with the goal of assessing successful treatment or premature relapses. This technique has been used to measure parasite load and evaluate antileishmanial drugs in the *in vivo* mouse model (Bretagne et al., 2001; Nicolas et al., 2002; Garnier et al., 2007; Reimão et al., 2011) and, more recently, in the *in vitro* model (Ordóñez-Gutiérrez et al., 2009).

The present study validated the use of qPCR as a sensitive technique to accurately measure the activity of selected antileishmanial drug in an intracellular model. The detailed protocol was described for the first time, enabling medium-to-high throughput screening into laboratory workflow with the possibility of automated applications in DNA purification and PCR setup. This detailed protocol can also be applied to determine parasite load in therapeutic studies with intracellular models infected with different *Leishmania* species.

The *Leishmania* qPCR assay performance was strictly measured to ensure its validity. A mean standard curve derived from five independent experiments demonstrated high PCR efficiency (99.9%). This mean standard curve was linear over an at least 6-$\log_{10}$ range of DNA concentrations (the highest [2.54 10^8] to the lowest [2.54 10^3] quantifiable copy number established for the linearized plasmid DNA with the SSU rRNA 67-bp fragment), with a correlation coefficient of 0.99. The precision measured by inter- and intra-assay variability (reproducibility and repeatability, respectively) was also extremely satisfactory. Evaluation of the analytical sensitivity of the *Leishmania* qPCR assay showed that it could accurately detect 2.54 10^2 copies of linearized plasmid DNA, corresponding to 1.5 *Leishmania* cells. In another approach,

Table 1
Comparative IC_{50} and IC_{90} of amphotericin B against intracellular *L. infantum* in an amastigote-macrophage assay, as determined by sigmoid dose–response curves.

	Methods		
	Microscopic counting[a]	qPCR assay[b] *amastigotes-100 macrophages*	qPCR assay[b] *Leishmania* DNA load
IC_{50} (μg/mL)	0.02	0.02	0.02
(CI 95%)	(0.01–0.03)	(0.01–0.03)	(0.01–0.04)
IC_{90} (μg/mL)	0.04	0.04	0.04
(CI 95%)	(0.01–0.15)	(0.001–0.98)	(0.002–0.76)

[a] Mean of the results of three independent experiments, each in triplicate.
[b] Mean of the results of two independent experiments, each in triplicate.

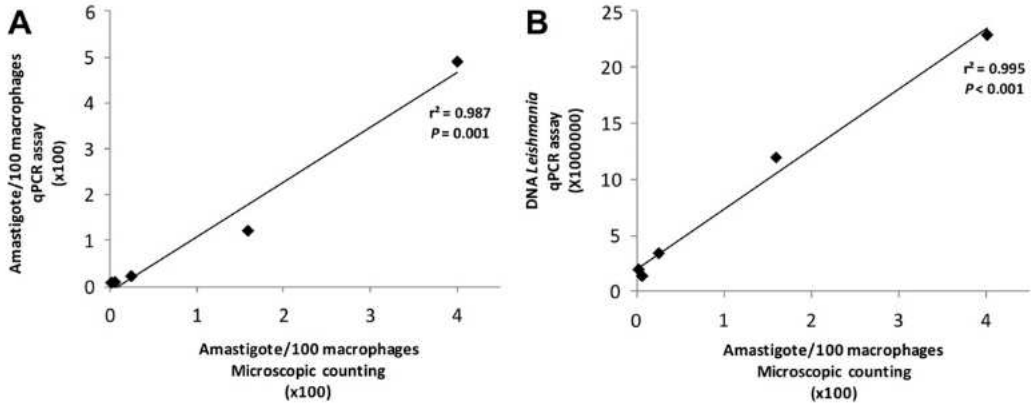

Fig. 1. Correlation between parasite load estimated by microscopic counting and the qPCR assay in an amastigote-macrophage model to assess *in vitro* amphotericin B activity against *L. infantum*. (A) A Pearson's correlation coefficient of 0.987 ($P = 0.001$) was found for a comparison considering two independent experiments and the values of the parasite load determined by microscopic counting and the qPCR assay. The results were expressed by the number of amastigotes-100 macrophages. (B) Considering the correspondence between microscopic counting and the qPCR assay with the *Leishmania* DNA load results, a Pearson's correlation coefficient of 0.995 ($P < 0.001$) was observed. The continuous line represents the linear regression.

the analytical sensitivity was evaluated as the capability to detect *L. infantum* genomic DNA, and the result obtained (0.036 pg) corresponds to less than a single parasite cell.

The potential to evaluate known antileishmanial substances was tested using amphotericin B in an *L. infantum* amastigote-macrophage model. The results of two experiments showed a dose-dependent effect on parasite load, with an IC_{50} value of 0.02 μg/mL and an IC_{90} value of 0.04 μg/mL. The same parasite load kinetics were observed after the assessment of three independent experiments with the classical method of microscopic counting using Giemsa stained coverslips (Fig. 1), and exactly the same IC_{50} and IC_{90} values were observed (Table 1). These data revealed the accuracy and sensitivity of the *Leishmania* qPCR assay when performed with fewer replicates. Additionally, the protocol developed was thoroughly tested, with special attention given to the removal of the macrophages from the coverslips, the total DNA extraction method applied and the analysis of the parasite load after qPCR evaluation. All of these efforts culminated in a highly accurate assessment of parasite load by *Leishmania* DNA content, as seen in Fig. 1B. The mouse qPCR assay with the reference gene ACTB was used to normalize data as well as to internally control for error in qPCR assays (Huggett et al., 2005).

The advantages and disadvantages in using DNA and RNA detection of the parasite in qPCR assays have been especially addressed when the objective is the monitoring of treatment in which the detection of viable parasites is crucial. For Reimão et al. (2011), DNA evaluation would not guarantee detection of living parasites, since available drugs are known to act progressively over a long period of time, resulting in a mixed population of living and dead organisms. Additionally, the proper chemical stability of the DNA molecule could hamper its degradation. Prina et al. (2007) using an *in vitro* model with mouse macrophages and L-leucine ester as a molecule that rapidly kills intracellular *L. amazonensis* amastigotes proved that kinetoplast and nuclear parasite DNA degradation occur very rapidly after amastigote death. Their conclusion was that qPCR assay with DNA evaluation assessed the presence of viable parasites, and it represents a robust method for diagnosis, and also for monitoring parasite reduction rate in either animal models or drug-treated patients. Although it was out of the scope of the present study to assess parasite viability, our choice to work with DNA was also influenced by clinical experience and technical feasibility. Previous clinical studies of our group using conventional PCR (Disch et al., 2004) and, more recently, parasite kinetics studies with qPCR (unpublished observation) showed a rapid clearance of circulating *Leishmania* DNA in peripheral blood after treatment of patients with visceral leishmaniasis. Sudarshan et al. (2011) highlighted that qPCR assay using kinetoplast DNA-specific primers was an important tool to estimate parasite kinetics in the blood of patients during treatment, and the test was used to detect cure and early relapses. Moreover, when working with RNA, a ribonuclease-free environment must be created, and one more step (cDNA synthesis) must be added to the assay, making it less practical for the medium-scale drug screening purpose.

In conclusion, qPCR proved to be an accurate method to assess parasite load in an amastigote-macrophage model. In contrast to the traditional method of expressing parasite number per macrophages, qPCR can be performed in a standardized medium-to-high throughput procedure with no subjective evaluation.

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