



# Absolute quantification of gene expression in drug discovery using RT-qPCR: Case of a drug used in the treatment of leishmaniasis

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## ABSTRACT

Leishmaniasis is a neglected disease and a public health concern. Chemotherapeutic agents available for the treatment of parasitic infections, including leishmaniasis, have several limitations. For that, we designed a highly sensitive assay using RT-aqPCR to evaluate the efficacy of antileishmanial drugs using SYBR Green to quantify the expression of marker genes. A matrix of reactions using different annealing temperatures and primer concentrations was tested to obtain optimum assay performance. The standard curves designed for quantification of parasites and macrophages showed linearity over a 9-log DNA concentration range. The amount of input target sequence was determined by plotting the  $C_t$  value of drug-exposed cells on the standard curves. We then tested the efficacy of miltefosine against *Leishmania tropica*. The RT-aqPCR assay was more sensitive, reproducible, and time-efficient than the conventional microscopic counting method. Most of the anti-parasitic drugs available have significant drawbacks, and there is an urgent need to develop new alternatives. Our assay expedites pre-clinical testing efficacy of candidate anti-parasitic compounds.

## 1. Introduction

Leishmaniasis is a vector-borne disease with a wide variety of clinical manifestations (Misra and Srivastava, 2020). Infections caused by the flagellate protozoan *Leishmania* spp. (Protozoa, Kinetoplastida, Trypanosomatidae) are described in both animals and humans (Reithinger et al., 2007). Due to its high prevalence, where two million new cases are reported annually, human leishmaniasis was classified as a neglected disease (World Health Organization, 2015). >20 *Leishmania* species are responsible for three different forms of leishmaniasis i) cutaneous leishmaniasis, the most common form of the disease, generates skin lesions, ii) visceral leishmaniasis, also known as kala-azar, causes fever, weight loss, enlargement of the spleen, and anemia, and iii) mucocutaneous leishmaniasis, mainly found in Latin America, leads to the partial or total obliteration of mucous membranes of the nose, mouth, and throat (Bern et al., 2008). Although infections frequently develop

under poor sanitary conditions, leishmaniasis is categorized as endemic in >100 countries and distributed across all continents, except Antarctica (World Health Organization, 2015). The life cycle of *Leishmania* occurs within two different hosts (phlebotomine sandflies and mammals) and can be divided into two developmental stages: promastigotes and amastigotes (Sunter and Gull, 2017). Promastigotes of *Leishmania* live exclusively in the gut of the infected phlebotomine female sand fly and can be injected into mammals during a blood meal (Gossage et al., 2003). Promastigotes are then phagocytized by macrophages and other mononuclear phagocytic cells. Intracellularly, the promastigotes are transformed into amastigotes, the tissue stage of the parasite (Gossage et al., 2003). Subsequently, amastigotes multiply by simple division and progress to infect other mononuclear phagocytic cells (Jamal et al., 2020).

Several reviews discussed the recent advances and new treatment options against leishmaniases (Andrade Neto et al., 2018; Roatt et al.,

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2020). The minimal therapeutic drug arsenal includes, but is not limited to, the conventional anti-leishmanial drugs such as pentavalent antimonials, amphotericin B, miltefosine, paromomycin, and pentamidine (Andrade Neto et al., 2018; Roatt et al., 2020). However, the available medications are limited and insufficient for the management of the disease due to the emergence and spread of drug resistance as well as the adverse side effects (Ponte-Sucre et al., 2017). Trials to assess the potential effectiveness of new therapeutics are ongoing. One of the conventional methods used to determine the therapeutic index of anti-leishmanial drugs is labor-intensive and relied on quantifying the parasite numbers using a hemocytometer (Moraes et al., 2008). This subjective method of analysis is not practical for high throughput screening models (Croft et al., 2006; Sereno et al., 2007; Suman Gupta, 2011). A better alternative would be through colorimetric assays used to determine the number of viable cells (Zghair, 2017), but the low sensitivity, chemical interference, and toxicity are considered major drawbacks (Wang et al., 2010). Fluorometric assays are superior to colorimetric assays in terms of sensitivity and specificity (Hemmilä and Stuart, 1997), with chemical interference and false positive results being among the reported limitations (Zhang and Guangwei, 2021). Molecular techniques were developed to surpass conventional drug discovery approaches and were widely adopted to find anti-parasitic agents. One such approach is absolute quantification (aq), which is considered a standard technique to investigate the nucleic acid copy number within tested samples. It's based on developing a standard curve using standards of known DNA concentrations (Dhanasekaran et al., 2010). A precise estimate of a target gene abundance can be calculated using its cycle threshold (Dhanasekaran et al., 2010). Absolute quantification of gene expression has also received much attention following major modifications introduced to the first developed approach (Leong et al., 2007). In the newly developed methodology cloning is not needed to prepare the standards for comparative quantification (Leong et al., 2007; Whelan et al., 2003). Instead, dsDNA purified from conventional PCR reactions were used as standards (Leong et al., 2007). However, to our knowledge, absolute quantification of gene expression using reverse transcription quantitative PCR (RT-aqPCR) was not considered or tested as a possible means to evaluate the efficacy of drugs against parasites. In this study, we aimed at i) investigating the sensitivity and the reproducibility of RT-aqPCR for determining the activity of drugs against promastigotes, axenic, and intracellular amastigotes of *L. tropica* as well as macrophages and ii) comparing the half maximal inhibitory concentration (IC<sub>50</sub>) needed to kill 50% of the parasite and macrophage populations as determined using RT-aqPCR and microscopic counting.

## 2. Materials and methods

### 2.1. Ethical approval

*Leishmania tropica* LT2 (strain designation: MHOM/LB/2015/IK) was originally isolated in 2014 from skin punch biopsies collected at the American University of Beirut Medical Centre (AUBMC) with detailed patients' annotations as previously described (Salloum et al., 2020). The biopsy specimens were obtained after an informed consent form was secured from each individual according to an approved protocol by the Institution Review Board (IRB) at the American University of Beirut Medical Centre (AUBMC) (approval reference #PALM I.K.01) (Salloum et al., 2020). All patients completed a risk assessment form. Experiments involving human research participants have been performed in accordance with the Declaration of Helsinki. The obtained ethical approval was also processed and accepted at Newcastle University (Ref: 9663/2016) (Salloum et al., 2020).

### 2.2. Chemicals

Miltefosine 98%, one of the most utilized anti-leishmanial drugs, was purchased from Sigma-Aldrich (CAS Number 58066–85-6). The drug

was stored according to the manufacturer's recommendations until further use. One million macrophages and parasites were each exposed to different concentrations of miltefosine (0.01, 0.05, 0.1, 0.5, 1, 2, 5, 7, and 10 µM) for 24 h.

### 2.3. THP-1 macrophages

THP-1 cells were maintained in standard RPMI-1640 medium (Sigma # R8758) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Thermo # 10500064) as well as 1% penicillin-streptomycin (Lonza # 17-602E) and incubated at 37 °C in total darkness. The cells were induced to become adherent, having a mature macrophage-like phenotype, in 6-well plates (TPP # 92006) by the addition of 50 ng/mL phorbol 12-myristate 7-acetate (PMA) from a stock of 1 mg/mL (Fisher # BP685–1) followed by overnight incubation. PMA-treated adherent THP1 (P-THP1) cells were washed three times with PBS and cultured in a fresh medium. Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Invivogen # TLRL-EBLPS) was added at 1 ng/mL for 4 h to each well to stimulate the macrophages.

### 2.4. Parasite culture and maintenance

*Leishmania* promastigotes were maintained in standard RPMI-1640 medium (Sigma # R8758) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Thermo # 10500064) and 1% penicillin-streptomycin (Lonza # 17-602E) and incubated at 25 °C in total darkness. *Leishmania* promastigotes were used to initialize axenic amastigotes as previously described (Teixeira et al., 2002). To culture intracellular amastigotes, 1 million THP-1 macrophages were seeded in 6-well-plates, as described above. Promastigotes were co-incubated with the macrophages at a parasite/macrophage ratio of 10/1. The infection was allowed to proceed overnight at 37 °C with 5% CO<sub>2</sub>. The cells were then washed two times with PBS to remove non-internalized promastigotes. Infected cells were incubated for an additional 24h to establish the infection.

### 2.5. Microscopic counting

The numbers of drug-exposed promastigotes, axenic amastigotes, and macrophages were measured using a Neubauer bright-line hemocytometer (Blaubrand®, Germany) under a light microscope. For intracellular amastigotes, the cells adhered to the slides were allowed to dry, fixed in methanol, and stained with 10% Giemsa. After that, the number of amastigotes was counted under a light microscope. Half maximal inhibitory concentration (IC<sub>50</sub>) and their 95% interval confidence limits were calculated.

### 2.6. Absolute quantification of gene expression

#### 2.6.1. RNA extraction

Total RNA extraction from treated, and non-treated control cells was performed using the RNeasy mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted RNA samples were subjected to DNase I (Fermentas, #EN0521) treatment to eliminate genomic DNA carryover. DNA digestion was conducted at 37 °C for 30 min. The samples

were then mixed with 70% ethanol, and purified with RNeasy columns. RNA concentration and purity were determined using Nanodrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). The quality of the RNA was verified by electrophoresis on a 1.5% agarose gel, and the extracted RNA was stored at –80 °C. RNA (2 µg) was reverse transcribed into cDNA using a Revert Aid First cDNA Synthesis Kit (#K1622-Thermo Scientific). The resulting cDNA was stored at –30 °C until analyzed by RT-aqPCR.

### 2.6.2. RT-aqPCR analysis

To investigate the activity of miltefosine against parasites and macrophages a fragment of the minicircle kinetoplast DNA (kDNA) and GAPDH were amplified using a set of primers as shown in Table 1, and BIO-RAD thermocycler (CFX96 Real-Time System, BIO-RAD, USA). SYBR® Green 2× (Sigma Aldrich) was used to obtain RT-aqPCR products. To establish efficient amplification and quantification of the target sequence, the RT-aqPCR protocol was optimized using a matrix to evaluate primer concentrations and the annealing temperature. The objective was to find the combination that will yield a robust assay while reducing non-specific amplification and primer dimers. Using the optimization matrix, different concentrations of the forward and reverse primers (50, 150, 300, and 600 nM) were evaluated for amplification performance. In parallel, a PCR temperature gradient, 55.2–65.2 and 51.8–61.8 °C was used to determine the optimum annealing temperature to amplify kDNA and GAPDH, respectively. The gradient was selected to cover  $\pm 5$  °C of the lowest calculated melting temperature for each primer set (Table 1). The amplification reactions were performed in a 25  $\mu$ L reaction mixture having 12.5  $\mu$ L of SYBR, each of the forward and reverse primers at optimal concentration, 9.5  $\mu$ L nuclease-free water, and 1 ng cDNA. The following was the used RT-aqPCR protocol: 1.5 min activation/denaturation step at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at the optimum annealing temperature, and 30 s at 72 °C. Melting curve analysis (single peak) was used as an indicator of the amplification specificity. Negative controls (no template) were also used to detect primer dimerization and nonspecific amplification. All reactions were duplicated and repeated three times, and mean values were calculated for the final analysis. The amplification efficacies of the test samples (drug-exposed *L. tropica* and macrophages) were estimated by plotting the  $C_t$  values against the log of input nucleic acid (10-fold serial dilutions) and calculated as  $E = 10^{[-1/\text{slope}]}$ , using the slope of the curves. Concentrations reducing 50% of gene expression (aqIC<sub>50</sub>) were calculated.

### 2.6.3. Standard curve

To enable the quantification of PCR products, standard curves, for *L. tropica* (kDNA) and macrophages (GAPDH), were generated as previously described (Leong et al., 2007). In brief, conventional PCR amplifications were performed in a total volume of 20  $\mu$ L, including the following: 10  $\mu$ L PCR Master Mix 2× Thermo Scientific, Massachusetts, USA (Taq DNA polymerase (0.05 U/ $\mu$ L), reaction buffer, 4 mM MgCl<sub>2</sub>, 0.4 mM of each dNTP), 1 ng of cDNA, the optimum concentration of the above-mentioned primers, and 7  $\mu$ L of distilled water. A 30-cycle PCR amplification was then performed according to the following cycling conditions: 95 °C for 3 min and, 30 cycles at 95 °C for 30 s, optimum annealing temperature for 30 s, 72 °C for 30 s, and a final elongation step at 72 °C for 1 min. PCR products were visualized on a 1.5% agarose gel. PCR products were then purified using the QIAquick PCR Purification kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and sequenced using either the forward or reverse primer on ABI3500 iGenetic analyzer as described (Nemer et al., 2006).

The PCR products were also used to generate standard curves for absolute quantification. The concentration and purity of the dsDNA were determined at 260 nm and 260/280 nm using the NanoDrop (NanoDrop Technologies, Wilmington, DE, USA).

Serial 10-fold dilutions of the purified dsDNA were re-amplified as shown above. An aliquot of each dilution ( $1 \times 10^{-1}$ – $10^9$  copies) in

triplicates was used as templates in RT-aqPCR. The number of molecules in the purified product was calculated using Avogadro's constant via the online tool from URI Genomics and Sequencing Center (<http://cels.uri.edu/gsc/cndna.html>). Standard curves were generated by plotting the log of the purified dsDNA concentration against its measured  $C_t$ . The RT-aqPCR assays were performed in triplicates. The melting curve analysis (single peak) was used to confirm the production of single and specified products. To detect primer dimerization, negative controls (no DNA template) were exhibited. The amplification efficacies of the standard solution (purified dsDNA of *L. tropica* and macrophages) were calculated as stated above.

### 2.7. Statistical analysis

IC<sub>50</sub> and aqIC<sub>50</sub> were calculated using the probit regression analysis in SPSS. The data obtained were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, Ill., USA). The Tukey test was used at the 5% threshold for the separation of means. A *P*-value of  $\leq 0.05$  was considered significant. The intraassay coefficient of variation was calculated to analyze the reproducibility of the RT-aqPCR assay using SPSS.

## 3. Results

### 3.1. RT-aqPCR development

*Leishmania* (promastigotes, axenic and intracellular amastigotes) and macrophages were subjected to RT-aqPCR analysis targeting kDNA and GAPDH, respectively, as target genes to determine expression post-drug exposure. Quantification of kDNA and GAPDH expression was used to assess the therapeutic index of miltefosine against *Leishmania*. kDNA and GAPDH were successfully amplified by conventional and RT-aqPCR, and the primer concentrations (forward and reverse) and annealing temperatures showing the lowest  $C_t$  were subsequently chosen. Clear single bands with high specificity and expected amplicon sizes (116 bp for kDNA and 128 bp for GAPDH) were obtained for kDNA using a final concentration of 600 nM forward and 300 nM reverse primer and annealing temperature of 59.2 °C, while it was for GAPDH 300 nM for each forward and reverse primer and annealing temperature of 58.2 °C (Fig. 1). The melting temperature for kDNA and GAPDH was 82.3 and 83 °C, respectively. The no-template-control dissociation curve showed a flat profile.

The test samples (miltefosine-exposed cells) and the standard solutions (purified dsDNA) were subjected to RT-aqPCR analysis targeting kDNA and GAPDH to quantify the abundance of *L. tropica* and macrophages, respectively. The number of targeted sequences throughout the PCR reaction cycles was measured constantly. PCR products were monitored by measuring the emitted fluorescence from SYBR-green after every cycle. The  $C_t$  values of the test samples were deduced/dsDNA copies and are shown in Supplementary Table (S1). The obtained standard curves were used to determine amplicon copy numbers in the test samples.  $C_t$  values for standards ranging from 10 to  $10^9$  dsDNA copies of kDNA and GAPDH fell along a straight semi-log trendline with  $R^2$  values of 0.9989 and 0.9998, respectively (Fig. 2). The slope was used to determine the amplification efficiency using the following formula:  $E = 10^{[-1/\text{slope}]}$  (Fig. 2). Accordingly, the amplification efficiency was

**Table 1**  
List of primers.

Primer	Sequence 5' → 3'	Amplicon size (bp)	Melting temperature	Amplification efficiency (%)	Reference
kDNA Forward Primer	CCTATTTTACACCAACCCCACT	116	60.25	98.04	(Yehia et al., 2012)
kDNA Reverse Primer	GGGTAGGGGCGTTCTGCGAAA		65.01		(Yehia et al., 2012)
GAPDH Forward Primer	TCCACCTTTCTCATCCAAG	128	56.82	101.4	This study
GAPDH Reverse Primer	CATCACCCCTCTACCTCCCT		59.73		This study

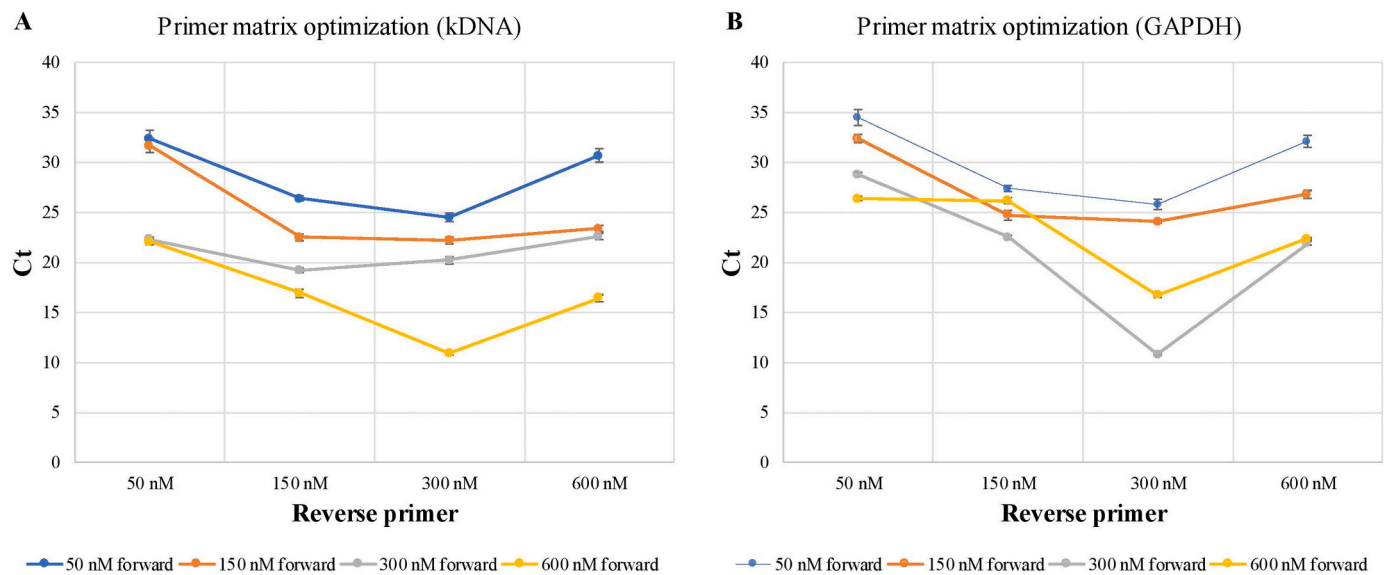


Fig. 1. Primer optimization matrix data.  $C_t$  ( $\pm$  S.E.) of each primer concentration pair at the optimal annealing temperature (A: 59.2 °C and B: 58.2 °C) are plotted.

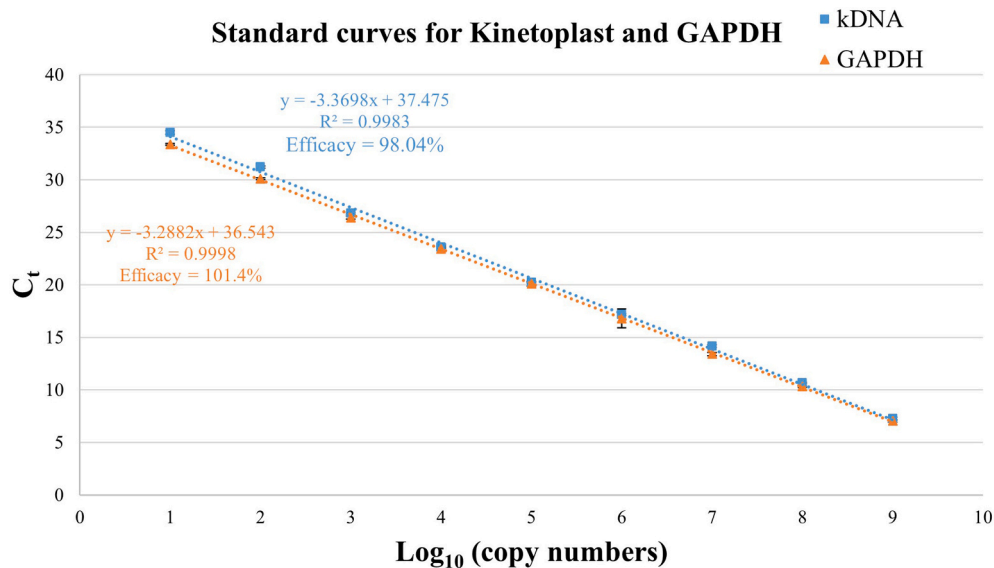


Fig. 2. Standards ( $10^{-10}$ ) plot for kDNA and GAPDH from  $C_t$  values of standards.  $R^2$  value of 0.9983 and 0.9998 for kDNA and GAPDH, respectively. Amplification efficiency was 98.04% for kDNA and 101.4% for GAPDH based on the following formula:  $E = 10^{[-1/\text{slope}]}$ .

Table 2

Mean aqIC<sub>50</sub> and IC<sub>50</sub> and their 95% confidence limits from promastigotes, axenic amastigotes, intracellular amastigotes, and macrophages values obtained with aqPCR and microscopic counting.

	Repetition	Promastigotes			Axenic amastigotes			Intracellular amastigotes			Macrophages		
		Value ( $\mu$ M)	Lower bound ( $\mu$ M)	Upper bound ( $\mu$ M)	Value ( $\mu$ M)	Lower bound ( $\mu$ M)	Upper bound ( $\mu$ M)	Value ( $\mu$ M)	Lower bound ( $\mu$ M)	Upper bound ( $\mu$ M)	Value ( $\mu$ M)	Lower bound ( $\mu$ M)	Upper bound ( $\mu$ M)
aqIC <sub>50</sub> (aqPCR)	1st	1.2	0.5	1.8	1.19	0.67	1.71	2.01	0.76	5.58	75.96	53.25	90.97
	2nd	1.64	1.18	2.1	1.07	0.67	1.48	2.43	0.87	7.7	72.83	48.89	87.32
	3rd	1.58	1.21	1.91	0.7	0.4	1.02	2.36	0.92	6.72	71.75	45.74	85.2
	Mean	1.47 <sup>a, *</sup>	0.96	1.93	0.98 <sup>a</sup>	0.58	1.4	2.26 <sup>a</sup>	0.81	6.67	73.51 <sup>a</sup>	49.29	87.83
IC <sub>50</sub> (Microscopic counting)	1st	1.58	0.5	2.86	0.89	0	1.56	2.12	0.79	4.99	71.99	41.34	83.68
	2nd	1.54	0.49	2.82	0.88	0	1.46	2.26	0.77	5.14	73.14	44.8	84.95
	3rd	1.56	0.47	2.86	0.89	0	1.57	2.27	0.85	5.23	72.93	46.28	84.61
	Mean	1.56 <sup>a</sup>	0.48	2.84	0.89 <sup>a</sup>	0	1.53	2.21 <sup>a</sup>	0.81	5.13	72.68 <sup>a</sup>	44.14	84.41

\*†Values followed by the same letter in the same column are not significantly different at the 5% threshold.



98.04% for kDNA and 101.4% for GAPDH, while it was 97.2% and 101.81% for kDNA and GAPDH, test samples respectively. Using RT-aqPCR we were able to detect *L. tropica* and macrophages in all the test samples. In contrast to the conventional microscopic-based approach, it was possible through RT-aqPCR to detect dsDNA from test samples exposed to the highest concentrations of the drug (as low as 2.6 dsDNA copies/test sample could be detected) (Supplementary Table S1). The dsDNA copies found in the test samples were in the range of ~3 to 80,000,000. The highest detected rate of gene expression was measured in the controls (untreated cells of *L. tropica* and macrophages). The rate decreased in a dose-dependent manner.

### 3.2. Sensitivity of RT-aqPCR

The median lethal concentrations are presented in Table 2. In all test samples, no statistically significant differences were found between RT-aqPCR (aqIC<sub>50</sub>) and microscopic counting (IC<sub>50</sub>) for promastigotes ( $F = 0.415$ ,  $df = 1$ ,  $P > 0.05$ ), axenic amastigotes ( $F = 0.415$ ,  $df = 1$ ,  $P > 0.05$ ), intracellular amastigotes ( $F = 0.130$ ,  $df = 1$ ,  $P > 0.05$ ), and macrophages ( $F = 0.395$ ,  $df = 1$ ,  $P > 0.05$ ).

### 3.3. Reproducibility of RT-aqPCR

To analyze the reproducibility of the RT-aqPCR assay, we used the intraassay coefficient of variation. Three replicates of nine 10-fold dilutions (10–10<sup>9</sup> copies per reaction) were assessed. The intraassay variations of aqIC<sub>50</sub> among replicates were 0.16, 0.26, 0.1, and 0.03% for promastigotes, axenic amastigotes, intracellular amastigotes and macrophages, respectively.

## 4. Discussion

RT-qPCR is an indispensable technique for the investigation of microorganisms of medical (Jauregui et al., 2001), environmental (Ettenauer et al., 2014), agricultural (Gao et al., 2004), and alimentary (Hein et al., 2001) interest. However, evaluating the efficacy of drugs against pathogens via the traditional approaches is time-consuming and has low specificity. In this study, we developed a new molecular qRT-PCR-based protocol to assess the therapeutic index of antiparasitic drugs. Our developed protocol was designed to quantify using RT-aqPCR and SYBR green. The approach showed high sensitivity and reproducibility in assessing gene expression over a wide range of cell concentrations. It's noteworthy that Nicolas et al. (2002) previously used RT-qPCR to detect *Leishmania* and amplified a 116-bp fragment from minicircles of the kinetoplast DNA. They used the genomic DNA of *L. tropica* to quantify the relative burden of the parasite in mouse tissues. Using this assay, they were able to detect *L. tropica* with sensitivity, which was attributed to the high copy number of kDNA/parasite. This approach was also used for the diagnosis and monitoring of canine leishmaniasis (Francino et al., 2006) and the detection with high sensitivity *L. infantum* in blood samples from patients with Mediterranean visceral leishmaniasis (Mary et al., 2004). Also, a study conducted by Gomes et al. (2012) showed that absolute DNA quantification is reliable to assess the parasite load in amastigote-macrophage assays. This correlates favorably with our results and further supports the idea that molecular techniques are accurate and sensitive tools that can increase the throughput of drug screening. A major drawback of their method, however, is that PCR signals from dead parasites could be detected. It is very difficult to judge whether Ct values are derived from live or dead parasites. The authors suggested that kinetoplast and nuclear parasite DNA degradation occur swiftly after the death of the parasite and concluded that qPCR assay with DNA evaluation assessed the presence of viable parasites only. In our view, their findings are only conjectures based on a study carried out by Prina et al. (2007) who showed that the DNA of *L. amazonensis* amastigotes was rapidly degraded after exposure to 2 mM L-leucine methyl ester. However, the provided evidence was not conclusive since

the degradation of DNA could be modulated by numerous factors, including, but not limited to, the drug mode of action. To note, a comparative study held by Kulkarni et al. (2009) demonstrated that apoptotic (Class I) and nonapoptotic (Class II) antimicrobial peptides possess differential DNA degradation abilities. Therefore, the limitation of absolute DNA quantification is the inability to discriminate between dead and live cells, which could lead to false-positive results (Cangelosi and Meschke, 2014). To address this limitation, we relied on the proposed method for gene expression quantification of chosen marker genes. Leong et al. (2007) developed an innovative method to obtain standards for absolute quantification without molecular cloning by using purified dsDNA standards recovered by conventional PCR amplification of target sequences. This approach has the advantage of i) testing the specificity of the primers (through a single product), and ii) evading the need to use the “typical” gene-in-plasmid format and as such reducing the cost. RT-aqPCR additionally, doesn't require reference gene normalization (housekeeping) decreasing the error margin (Leong et al., 2007). However, the selection of the marker gene is crucial and impacts results precision and its use for adequate quantification should be under different experimental conditions (Dhedea et al., 2005). The marker gene expression should preferentially be independent of experimental perturbations and should not be modulated by drugs. In our study, kDNA and GAPDH were stably expressed when exposed to sub-lethal doses of the drug (data not shown), and we also detected similar amplification efficacies with the used dsDNA standards and the test samples.

Taken together, we hypothesize that miltefosine didn't interfere with the sensitivity and kinetics of the RT-aqPCR assay. A key concern regarding the reliability of our proposed approach and its utility for drug discovery would be amplification problems due to inhibiting substances. Schrader et al. (2012) reviewed the PCR inhibitors and their occurrence in different matrices and removal. Therefore the sensitivity of our approach should be tested using different matrices, especially those interfering with mRNA measurements in the test samples. In addition, it is important to highlight the usefulness of the assay to measure drug activity against different developmental stages of *L. tropica* (promastigotes, axenic, and intracellular amastigotes) and macrophages. No significant differences were detected between the median lethal concentrations obtained by microscopic counting (IC<sub>50</sub>) and RT-aqPCR (aqIC<sub>50</sub>) for all test samples (promastigotes, axenic amastigotes, intracellular amastigotes and macrophages). Also, the extremely low intra-assay coefficient further confirms the validity of the introduced assay. We also believe that the method developed in this study is cost-effective, fast, and can be customized to cover a wide range of therapeutic agents and organisms.

## 5. Conclusion

In this study, we developed, for the first time, a novel assay that can assess the efficacy of drugs against microorganisms and target cells. From this standpoint, we believe that this method will substitute or strengthen the currently used in vitro testing techniques paving the way for the development of new drugs. In the era of high throughput analysis and rapid data reporting, we propose the optimization of the used RT-aqPCR to cover a broad range of organisms.

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## Ethical approval

Not required.

## Consent to participate

Not applicable.

## Consent for publication

All authors have read and approved the manuscript for publication.

## Declaration of Competing Interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2022.10.012>.

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