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Major Article

Standardization and evaluation of a duplex real-time quantitative PCR for the detection of *Leishmania infantum* DNA: a sample quality control approach

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Abstract

Introduction: Molecular techniques have been shown to be alternative methods for the accurate detection of infectious and parasitic diseases, such as the leishmaniases. The present study describes the optimization and evaluation of a duplex real-time quantitative PCR (qPCR) protocol developed for the simultaneous detection of *Leishmania infantum* DNA and sample quality control. **Methods:** After preliminary tests with the newly designed TaqMan® probes for the two targets (*L. infantum* and glyceraldehyde 3-phosphate dehydrogenase (G3PD) gene), the duplex qPCR protocol was optimized. For the evaluation of the standardized protocol, human blood samples were tested (n=68) and the results were compared to those obtained by reference diagnostic techniques. Statistical analyses included percentage agreement and the Kappa (*k*) coefficient. **Results:** The detection limit of *L. infantum* DNA reached $2x10^2$ fg (corresponding to ~1 parasite) per µL of blood (ε: 93.9%). The percentage agreement obtained between the duplex VL qPCR and the reference techniques was individually obtained as follows: molecular: 88.3% (*k*=0.666; 95% CI 0.437–0.894, good), and serological: 81.7% (*k*=0.411; 95% CI 0.125–0.697, moderate). Between the reference techniques, the percentage agreement was 86.7% (*k*=0.586; 95% CI 0.332–0.840, moderate). **Conclusions:** The new duplex VL qPCR protocol indicated good potential for the accurate, fast, and reliable detection of *L. infantum* DNA, when applied as a complement to the classical diagnostic tools already available, especially in health or research reference centers.

Keywords: Visceral leishmaniasis. Diagnosis. HIV/VL co-infection. Sample quality control. Duplex qPCR.

INTRODUCTION

The leishmaniases are parasitic diseases caused by protozoans from the genus *Leishmania* (Kinetoplastida, Trypanosomatidae)[1]. In the Latin America, the development of visceral leishmaniasis is often associated with the species *Leishmania infantum* (*L. infantum*)[2].

To control the advancement of disease in infected individuals, early detection and fast implementation of treatment are crucial for successful outcomes. However, classic diagnostic methods have several limitations, such as low sensitivity and high invasiveness (parasitological tests), the possibility of cross-reactivity (as with *Leptomonas seymouri*, a monoxenous trypanosomatid)[3], and the lack of accuracy in diagnosing immunosuppressed patients (serology), such as those co-infected with human immunodeficiency virus and visceral leishmaniais (HIV/VL)[4,5]. Thus, these diagnostic methods can yield false positive and negative results, thus impairing the appropriate therapeutic intervention.

Given the limitations of classic diagnostic methods, molecular methods, especially polymerase chain reaction (PCR), have become popular alternatives for the diagnosis and control of Visceral Leishmaniais (VL)[4,6-10]. Specifically, real-time quantitative PCR (qPCR) has been widely used by many authors because of the technique's ability to quantify amplified genetic material and estimate the parasitic load, thus allowing researchers to study host-parasite interaction and monitor therapy efficacy and relapses[11]. However, this method also contains limitations that can produce false negative results, such as PCR inhibitors, including proteinase K and phenol, and the incorrect storage and loss of deoxyribonucleic acid (DNA) during the extraction step[8,12,13].

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To enhance the efficiency and reliability of diagnostic techniques, sample quality controls are often included that are based on the amplification of a host's constitutive genes, such as the β-actin, β-globin, albumin, and glyceraldehyde 3-phosphate dehydrogenase **(**G3PD) genes in mammals. Quality control measures have been routinely used in laboratories, but these additional steps generate more costs and prolong the time to result interpretation and reporting[8,9,11]. However, during qPCR, it is possible to simultaneously amplify both the sample quality control and the target DNA in the same tube by applying multiplex protocols that use probes directed at a specific target and marked with different fluorochromes[12,14].

The aim of this study was to standardize and evaluate the inclusion of a sample quality control (internal control) into a qPCR protocol for the detection of *L. infantum* DNA, thus enabling the simultaneous tracking of possible false negative results.

METHODS

Ethical considerations

Prior to sample collection, written informed consent was obtained from all subjects and/or their legal guardians. This work was approved by the Research Ethics Committee (CEP/CPqAM/FIOCRUZ-PE, 42/2010) in consonance with the National Research Ethics Committee (CONEP-BR; CAAE: 0041.0.095.000-10). All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Study design

This study evaluated diagnostic methods based on the steps proposed by Sackett and Haynes[15], which consists of the following three phases: I) analytical sensitivity analysis; II) reproducibility analysis; and III) concordance analysis between results of the new test and results obtained by reference tests, using samples from patients.

Sample collection, processing, and group definitions

Samples were obtained by convenience (Non-Probabilistic Sample)[16]. Blood samples (2-4 mL) were collected from healthy individuals living in non-endemic areas, who had not previously submitted for a blood transfusion and who were negative for immunological and molecular tests (negative control group). Blood was also collected from patients living in endemic areas and presenting with suggestive VL symptomatology. The patients were treated in the following reference hospitals in the Pernambuco state of Brazil: The Professor Fernando Figueira Integral Medicine Institute (IMIP); Correia Picanço Hospital (HCP); Oswaldo Cruz University Hospital (HUOC); Clinics Hospital (HC); and Barão de Lucena Hospital (HBL). All specimens were processed in laboratories of the Aggeu Magalhães Research Center (CPqAM-FIOCRUZ; Recife, PE, BR). Blood samples were extracted using the QIAamp® DNA Blood Mini Kit (QIAGEN Sample and Assay Technologies), according to the manufacturer's instructions.

Immunological and molecular tests

All individuals included in the study were submitted to the following reference assays: an immunological test for anti-*Leishmania* antibody detection through recombinant kinesin 39 - immunochromatographic test (rK39-ICT) (InBios, Seattle, WA, USA) was performed following manufacturer's instructions; while a qPCR molecular blood test for *L. infantum* kinetoplast DNA (kDNA) minicircle detection was performed as per the protocol previously described by Paiva-Cavalcanti et al[17].

Parasitological test

Some patients suspected of having VL underwent a parasitological test that included a bone marrow biopsy. Six bone marrow smears were prepared from collected biological specimens and tested for amastigote forms (methodology preconized by the Ministry of Health, Brazil). The aspirates were obtained by trained physicians from the respective reference hospitals and only under prescription.

Sample positivity criteria

Positive results for at least two of the reference techniques – VL qPCR, rk39-ICT, and bone marrow aspiration – were defined as the set of diagnostic criteria (characterizing VL cases). As criteria of positivity of the Singleplex qPCR assay, the amplification curve had to surpass the threshold before cycle 36, as recommended by Applied Biosystems[18]. The quality assurance of each sample was achieved in separated reactions by mammalian G3PD constitutive gene amplification, employing primers G1F (5'-ATC TTC CAG GAG CGA GAT CCC-3') and G1R (5'-AGG GAT GAC CTT GCC CAC-3')[8].

Development of the duplex qPCR assay

The duplex qPCR system was developed through the combination of the *L. infantum* primers (LINF 1B)[17] and the G3PD1 set[8] for the simultaneous detection of the *L. infantum* kDNA and the G3PD gene from mammals (internal control), respectively. All experiments were performed using the ABI Prism 7500 (Applied Biosystems®, CA, USA) equipment. The software ABI Prism 7500 SDS was used for the analysis, interpretation, and registration of results.

***TaqMan® probes design*:** using the software PrimerQuest (http://www.idtdna.com/scitooes), specific probes for the sets G3PD1 (probe A) and LINF 1B (probe B) were designed. To compose the duplex qPCR assay, the probes were chosen following the manufacturer's instructions for the TaqMan probe (Applied Biosystems®) technology. The probes' specificity was preliminarily analyzed by multiple alignments of sequences, using the nucleotide Basic Local Alignment Search Tool (BLASTn) (http://blast.ncbi.nlm.nih.gov).

***Individual optimization of the sets LINF 1B and G3PD1*:** preliminary singleplex qPCR was performed to determine the optimal amounts of primers and probes for the sets. First, between 5 and 25 pmol of the primers, G1F (forward), G1R (reverse), (G3PD1), and Linf.1-23F (5'-TCC CAA ACT TTT CTG GTC CT-3' forward), Linf.1-154R (5'-TTA CAC CAA CCC CCA GTT TC-3' reverse), and (LINF 1B), were tested

using the respective probe (A or B) at 12.5pmol/reaction. A standard amount of $1x10^6$fg of *L. infantum* (syn. *L. chagasi*) DNA (MHOM/BR/1974/PP75) or DNA extracted from whole blood (negative control group) was added to the respective reactions. The final volume per reaction was as follows: 50µL, consisting of 25µL TaqMan® Universal Master Mix (Applied Biosystems®, CA, USA) and 5µL of template. All samples were produced in duplicates. The cycling conditions used were the standardized cycles used by Paiva-Cavalcanti et al.[17]: 95ºC/15 s and 60ºC/1 min, at 40 cycles. The lowest amounts of forward and reverse primers that yielded a minimum C_t (threshold cycle) and a maximum ΔR_n (normalized reporter) were chosen as optimal. Second, between 2.5 and 12.5pmol of the probes A and B per reaction were tested by using the optimal amount of G3PD1 and LINF 1B primers found in the previous experiments. The same cycling and reaction conditions of the previous step, as well as the amount of the standard DNA, were utilized. The lowest amounts of the probes that yielded a minimum C_t were chosen as optimal.

Optimization of duplex qPCR system: the LINF 1B set was combined with G3PD1 set (primers + probe). The system formed (duplex VL qPCR) was evaluated in preliminary experiments by using the amounts of primers and probes optimized in the previous step and in the same cycling conditions that were standardized by Paiva-Cavalcanti et al.[17]. The detection limit was determined by using dilution curves prepared from the blood of healthy individuals (negative control group): concentrations between $2x10^{-1}$ and $2x10^5$fg (from 0.001 to 1,000 parasites, according to Grimaldi et al.[19], with a serial dilution factor of 10) per µL of whole blood genomic DNA from *L. infantum* (MHOM/BR/1974/PP75) were used. When necessary, changes were performed in the cycling conditions (annealing and extension temperatures), as well as in the concentration of reagents according to the Applied Biosystems® protocol[20].

Reproducibility analysis: for reproducibility evaluation of the new test, intra- and inter-assay analyses were performed. After optimization, DNA from two aliquots of three different concentrations ($2x10^2$, $2x10^3$ and $2x10^4$fg of *L. infantum* DNA per µL of blood) from the dilution curve was extracted. The duplex VL qPCR was performed with the duplicates (the two aliquots) of each selected concentration. The experiment was then repeated twice. The points in which the amplification curve surpassed the threshold (C_t values) were used to calculate the coefficients of variation (CV) between the replicates.

Comparative analyses

The samples were subjected to the duplex protocol and the results were compared with those obtained from the set of criteria (see item *2.6 Sample positivity criteria*), which was defined in this study as the set of diagnostic criteria. Comparative analysis between the techniques and concordance analysis was performed using descriptive statistics in absolute and percentage distribution values. Concordance was also evaluated by applying the Kappa (*k*) coefficient to the Confidence Interval (CI) set at 95%, and the agreement between the tests was judged using the Cohen[19] framework as follows: k=0.0, no agreement; 0.0≤k≤0.20, poor; 0.21≤k≤0.40, fair; 0.41≤k≤0.60, moderate; 0.61≤k≤0.80, good; and 0.81≤k≤1.00, very good. All analyses were performed with the BioEstat software (version 5.0; Mamirauá/CNPq, Belém, PA, Brazil). No template controls (NTC) and quantitative standards were included in all reactions.

RESULTS

Patient and group definitions

Blood samples from 68 patients who presented with symptoms indicative of VL (fever, hepatomegaly and/or splenomegaly, anemia, fatigue, and weight loss) were included in the analyses. Among them, 50 patients had been previously diagnosed with HIV/AIDS. Samples from 61 patients were subjected to the, rK39-ICT test. From these sample findings, only five patients underwent bone marrow aspiration for parasitological analysis (positive: 4; negative: 1). To maintain the reliability of the results, samples negative for the G3PD gene in the singleplex qPCR reaction (n=1) were excluded from the analysis of the duplex VL qPCR (total included, 60). According to the established gold standard, 8 (13.3%) patients were considered positive for VL *cases* and 52 (86.7%) patients were considered *non-cases* or negative patients.

Optimization of the duplex real-time PCR system

In regards to the requirements for the use of the probes, the sequences to each set of primers were designed. G3PD1 (5'-ATC ACT GCC ACC CAG AAG ACT GTG-3') was designed with the following characteristics: size, 24bp; GC, 54%; T*m*, 68°C; and reporter fluorochrome, VIC®. LINF 1B (5'-AAA TGG GTG CAG AAA TCC CGT TCA AA-3') was designed with the following characteristics: size, 26bp; GC, 42.3%; T*m,* 59.4°C; and reporter fluorochrome, FAM™. These sequences were analyzed *in silico*, demonstrating the impossibility of self-annealing and annealing with non-specific targets. Through singleplex qPCR reactions, the amounts (per reaction) of primers and probes were selected as follows: for G3PD1, 15pmol of each primer and 2.5pmol of probe A (C_t=30.88), and for LINF 1B, 10pmol of each primer and 7.5pmol of probe B [(C_t=13.04); **Figure 1**].

The duplex protocol was formed and evaluated in preliminary experiments that demonstrated the good performance of the two sets of primers, LINF 1B and G3PD1, for the simultaneous amplification of both targets. The added solution volumes of the primers and probes were adjusted to maintain the optimum amounts of reagents per reaction, for the multiplex format. An optimization procedure, referred to as the Limiting Primer Matrix, was executed according to the ABI Prism® 7700 Sequence Detection System (User Bulletin #*5*)[20], in an effort to improve the analytical sensitivity by minimizing the competition between the sets. The detection limit was then reassessed. After the modifications, the detection limit of the duplex VL qPCR was established: $2x10^2$fg of *L. infantum* DNA (~1 parasite, according to Grimaldi et al.[19]) per µL of blood; efficiency (ε), 93.9% (**Figure 2**). The duplex VL protocol was maintained in the same cycling conditions, as standardized by Paiva-Cavalcanti et al.[17]. Reaction conditions optimized for the duplex system

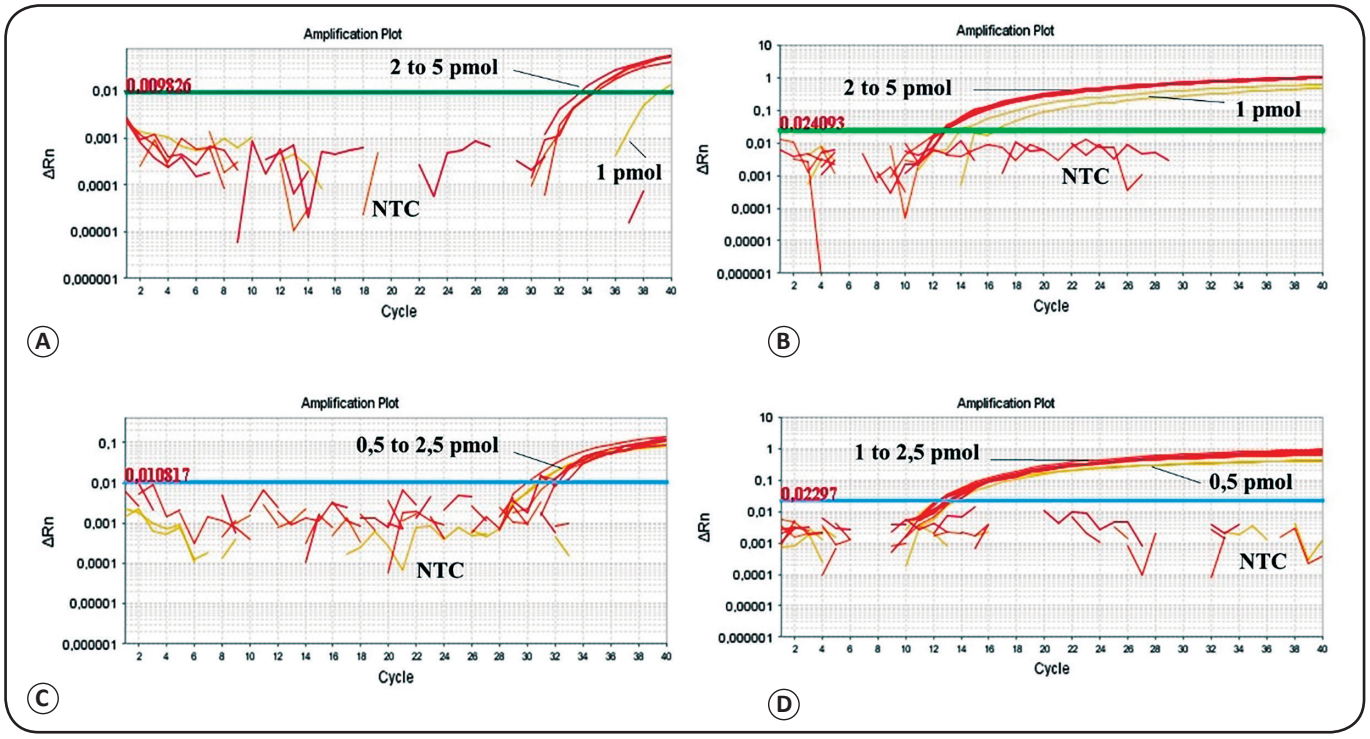

FIGURE 1 - Individual definition of the optimal amounts (per reaction) of primers and probe for each set. (A): G3PD1 primers: 15pmol. (B): probe A: 2.5pmol. (C): LINF 1B; primers: 10pmol, and (D): probe B, 7.5pmol. NTC: negative control. Optimal amounts of primers and probes were defined considering those with earlier threshold cycles (Ct). **ΔRn**: Reporter – normalized fluorescence; **NTC:** no template control (negative control); **G3PD1:** glyceraldehyde 3-phosphate dehydrogenase 1 primer set; **LINF**: *Leishmania infatum* primers set.

were developed as follows: G3PD1: 15pmol; LINF1B: 10pmol; probe G3PD1: 2.5pmol; and probe LINF1B: 7.5pmol. The volume of the TaqMan® Universal Master Mix 2X (Applied Biosystems) was 25µL. A volume of 5µL of DNA template was added. In total, the volume was 50µL.

Reproducibility analysis

The reproducibility analysis was conducted to determine the detection limit ($2x10^2$fg/µL) was maintained in both the intra- and inter-assays. The inter-assay CV, as calculated from the average C_t values of the duplicates of three different curve concentrations ($2x10^2$, $2x10^3$, and $2x10^4$fg per µL of blood) from three independent experiments, were as follows: 3.8%, 2.2%, and 2.8%, respectively. The intra-assay CV, as calculated from the C_t of the duplicates (from one experiment) of the same three curve concentrations, were as follows: 0.3%, 0.2%, and 4.4%, respectively.

Comparative analysis

The concordance analysis between the new protocol (duplex VL qPCR) and the set of diagnostic criteria (rK39-ICT + VL qPCR) showed an 81.7% agreement. Nevertheless, the *k* coefficient was considered *fair*: 0.373 (95% CI 0.081-0.665). The concordance analysis performed with the data submitted to the set of the diagnostic criteria (rK39-ICT + VL qPCR) and the bone marrow aspiration showed a 60% agreement. Since the number of patients who underwent bone marrow aspiration was low (n=5), the *k* analysis was not allowed.

To conduct a broader and more discriminative evaluation, comparative analyses were also individually performed with the reference techniques of the criteria set (**Table 1**). The duplex VL PCR and the rK39-ICT presented an 81.7% agreement with a *k* coefficient of 0.411 (95% CI 0.125-0.697). The percentage agreement between the duplex VL qPCR and VL qPCR was 88.33%, with a *k* coefficient of 0.666 (95% CI 0.437-0.894). The percentage agreement between VL qPCR and rK39-ICT (reference techniques was 86.7%, with a *k* coefficient of 0.586 (95% CI 0.332-0.840).

DISCUSSION

In recent years, molecular biology has been used in the development of alternative methods for the study and diagnosis of various infectious and parasitic diseases. The PCR technique and its variations have aided in the advancement of diagnosis accuracy in both clinical forms of leishmaniasis because the method enables a more sensitive and specific detection of the etiological agent's DNA in various samples, such as blood and urine[5,17,21-23].

As previously discussed, molecular techniques have numerous advantages, but they still contain some limitations, such as the occurrence of false negative results, as a result of using inadequate samples: presence of *Taq* polymerase enzyme inhibitors, such as proteinase K (used in DNA extraction process), high concentration of salts and ethylenediamine tetraacetic acid (EDTA), and poor storage. Through the habitual

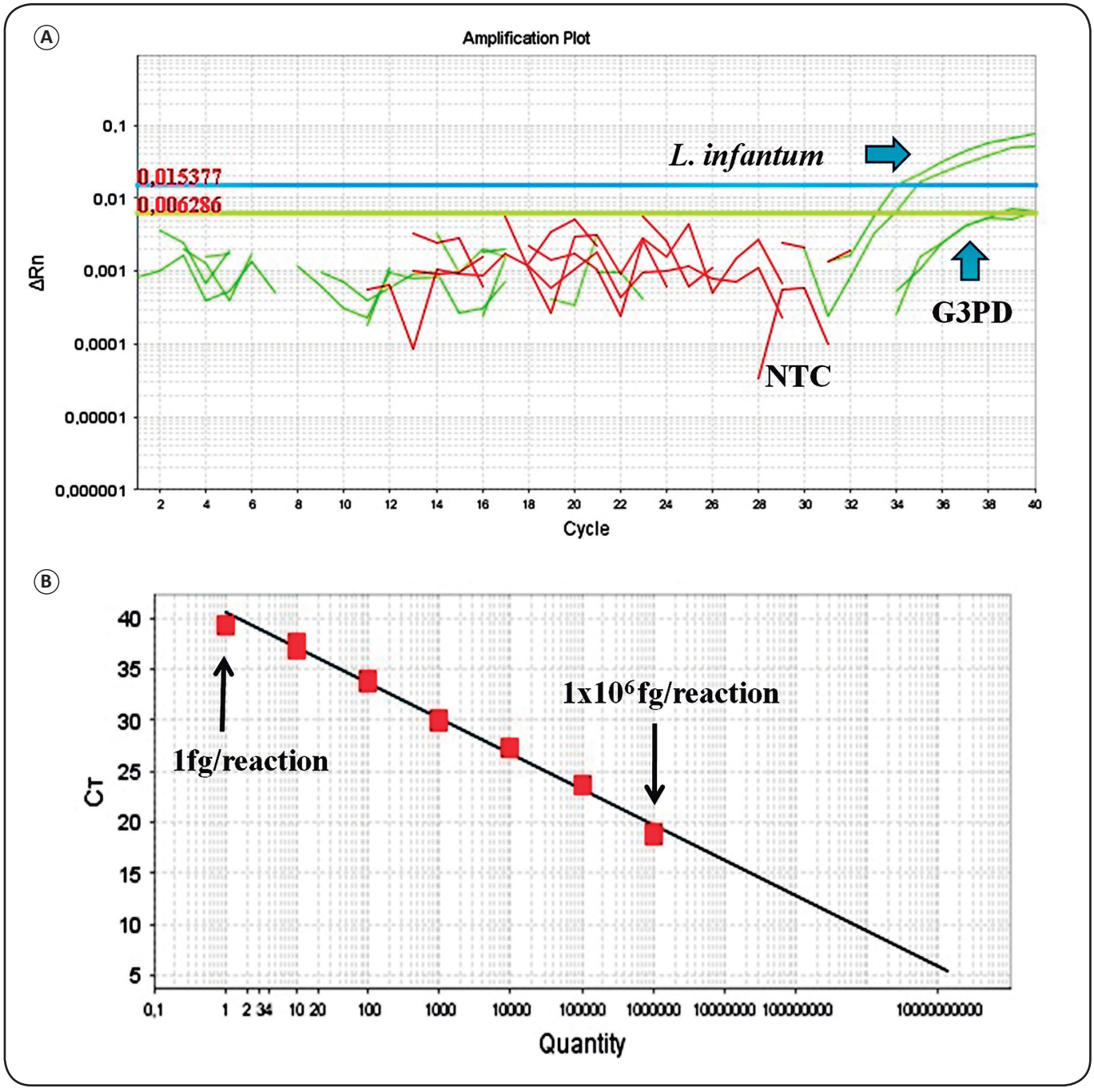

FIGURE 2 - Detection limit of the duplex VL qPCR. (A): Simultaneous amplification of *H. sapiens* genomic DNA by a set of G3PD1 primers, and *L. infantum* genomic DNA (MHOM/BR/1974/PP75) in the concentration of 2x10²fg per µL of blood by the LINF 1B primer. NTC: negative control. **(B)**: Standard curve of *L. infantum*, resulting from the detection limit experiment of the duplex VL qPCR. Quantities between 1fg and 1x10⁶fg of DNA per reaction (50µl) were used: Slope, -3,479; coefficient of determination (R^2), 0,991; and efficiency (ε), 93.9%. **CT:** Cycle threshold; ***L.*:** Leishamania; **G3PD1:** glyceraldehyde 3-phosphate dehydrogenase 1 primer set; **NTC:** no template control; **VL:** visceral leishmaniasis; **qPCR:** quantitative polymerase chain reaction; ***H.*:** *Homo*; **DNA:** deoxyribonucleic acid**; LINF**: *Leishmania infatum* primers set.

TABLE 1

Concordance analysis between the new duplex VL qPCR protocol and each reference technique employed for the diagnosis of VL.

Tests		**rK39-ICT**		**Total**
		Positive	Negative	
VL qPCR	Positive	8	2	10
	Negative	6	44	50
	Total	14	46	60
κ: moderate				
0.586 (CI 95% 0.332–0.840)				
Tests		**rK39-ICT**		**Total**
		Positive	Negative	
Duplex VL PCR	Positive	6	4	10
	Negative	7	43	50
	Total	13	47	60
κ: moderate				
0.411 (95% CI 0.125–0.697)				
Tests		**VL qPCR**		**Total**
		Positive	Negative	
Duplex VL PCR	Positive	10	4	14
	Negative	3	43	46
	Total	13	47	60
κ: good				
0.666 (95% CI 0.437–0.894)				

VL: visceral leishmaniasis**; qPCR:** quantitative polymerase chain reaction**; rK39:** recombinant kinesin 39; **ICT:** immunochromatographic test; **PCR:** polymerase chain reaction.

use of sample quality controls, predominantly based on the amplification of the host's constitutive genes, the chances of erroneous results in molecular diagnosis become smaller[8,24]. During real-time PCR reaction, the simultaneous amplification of a sample quality control and the target DNA in the same tube is only possible through the application of multiplex protocols that use probes that are marked with different fluorochromes and directed at the specific target, thus helping to reduce processing time and costs[8,12,14]. Therefore, this study aimed to develop and evaluate a duplex real-time PCR assay for VL diagnosis that could simultaneously detect *L. infantum* kDNA and the G3PD gene in blood samples to ensure the high quality of results through the association of accuracy and reliability.

From the individual optimization of the LINF 1B and G3PD1 sets, these probes excellently amplified their respective targets. Between the resulting C_ts and ΔR_n of the amounts of primers and probe evaluated in each set, minimal differences were observed (**Figure 1**). The optimization process of the duplex qPCR protocol promoted a good detection limit without major changes in reaction or cycling conditions in the protocol standardized by Paiva-Cavalcanti et al.[19]. Modifications in reagent concentrations and in cycling temperatures were performed to optimize the duplex VL qPCR; however, the detection limit was maintained ($2x10^2$fg of parasite DNA per µL of whole blood) and the analytical efficiency (ε=93.85%) had no significant improvement. As evidenced in **Figure 2**, the amplification of the parasite DNA is favored, and this is associated with the design of the probes, as well as with the chosen targets. The large amount of the host's genetic material that is simultaneously purified in the extraction step could impair the detection of the etiological agent DNA, mainly because of competition between the primer sets for the PCR reagents[9,25]. In larger parasite DNA concentrations, there is no amplification of the G3PD gene (as from the concentration of $2x10^3$fg per µL of sample), but this does not affect the validation of the results, since the intention of the reaction is to favor the target DNA appearance.

In this study, we evidenced and reinforced the importance of including sample quality control measures because in the non-amplification of this target, false-negative results are possible to track, thus avoiding the misinterpretation of results and increasing test reliability. Bezold et al.[13] detected potential false negative results in 20% of the samples tested in the molecular diagnosis of herpes simplex virus and the varicella-zoster virus using swabs; thus emphasizing the importance of using internal controls, especially when analyzing DNA from different types of clinical specimens. Gonçalves et al.[8] used the same sample quality control (G3PD gene) in multiplex reactions to detect VL through conventional PCR (cPCR) reactions, and demonstrated that it was possible to detect potential false negative results in 33% of the samples tested (no amplification of the G3PD gene). Gonçalves-de-Albuquerque et al.[25] standardized multiplex cPCR reactions for the diagnosis of VL in dogs, also using the G3PD gene as a quality control, and more than 15% of the samples were considered unsuitable for diagnostic definition because of no quality control amplification in the negative samples. In this study, all samples with negative results in the duplex qPCR protocol presented amplification of the quality control. Even though one inhibition in the singleplex qPCR was observed in this study, procedures that are established for good sample collection, storage, and processing must be rigorously followed.

The classical diagnosis for VL is based on parasitological and immunological techniques, and despite these techniques' widespread use, their existing limitations and potential for erroneous results demonstrate the need for a more thorough diagnostic scheme. According to Cota et al.[26] and Srividya et al.[27], the reliability of parasitological diagnostic techniques depends on numerous factors. The method is very specific, but sensitivity depends on good sample collection, quality, and preparation, in addition to the analyst's expertise. Singh and Sundar[28] indicate the difficulty and invasiveness of the collection procedure. Further, the parasitological diagnostic technique is not included in primary health care centers (PHC), making it difficult to access a high number of well diagnosed and characterized samples for evaluation. In this study, to increase the number of samples for the tests, the positivity criteria had to be elaborated upon based on well-established serological and molecular methods. The new duplex VL qPCR protocol showed a reasonable percentage agreement (60%) with microscopic examination of bone marrow aspiration. However, one of the patients had taken 18 doses of the antileishmanial N-methylglucamine antimoniate (Glucantime®, among 10 and 20 mg/Sb^{+5}/Kg/day) just after the positive result of the parasitological examination was found, thus causing both the singleplex VL qPCR and duplex VL qPCR results to be negative[22,29]. The three remaining patients who were positive for LV upon parasitological examination underwent treatment prior to sample collection; however, these patients had only taken one to two doses of the drug. In addition, the patient who was negative for LV upon bone marrow examination was positive for LV in both molecular techniques and rK39-ICT. By evaluating the parasitological test results within the predefined set of diagnostic criteria, the percentage agreement obtained remained 60%.

In the comparison of the duplex VL real-time PCR results and the results of the set of diagnostic criteria (rK39-ICT + VL qPCR), a good percentage agreement was reached (81.7%), despite the fair agreement obtained by the *k* coefficient (0.373; 95% CI 0.081-0.665). When compared to the original qPCR protocol standardized by Paiva-Cavalcanti et al.[17], the duplex VL real-time PCR technique had a great percentage agreement (88.33%), with a *k* coefficient indicating a good agreement (0.666; 95% CI 0.437-0.894) (**Table 1**). Only after the individual analysis was performed via the immunological technique (rK39-ICT) was it was possible to identify the likely reasons as to why there was a slight decrease in concordance between the duplex technique and the set of diagnostic criteria. Despite having low costs and quick rate of diagnosis, immunochromatographic rapid tests have some limitations that may promote erroneous diagnostic interpretation when the test is applied individually, such as low accuracy in immunosuppressed patients and cross-reactions with other trypanosomatids[4,30,31]. Thus, despite a moderate *k* agreement (0.411; 95% CI 0.125-0.697), only six out of the 13 samples were positive in both the duplex VL qPCR and the rK39-ICT (**Table 1**). All seven samples that presented negative in the immunological test, but positive in the duplex test, were from patients with symptomatic HIV. Further, the results of both the duplex and singleplex VL qPCRs were in agreement with one another in five of these seven samples.

In addition, there were important divergences between the results of the reference techniques, with *k* indicating moderate agreement [(0.586; 95% CI: 0.332-0.840); **Table 1**]. Naturally, methods with different principles (molecular and immunological) present discordant results when evaluated in the same population, thus highlighting the importance of adopting a reliable set of diagnostic criteria (associated with epidemiology plus clinical signs). In this context, the new duplex technique combined with classical diagnostic tools may help to develop accurate criteria for assessing positivity and minimize the occurrence of misdiagnosis.

Elmahallawy et al.[30] described the importance of using qPCR techniques because of the method's sensitivity, specificity, and quantitative ability, which enables the evaluation of the parasite load and treatment efficacy, especially in patients co-infected with HIV. Patients who are positive for HIV are particularly vulnerable to VL because the disease accelerates the replication and progression of HIV to AIDS and there is a higher risk of treatment failure and relapse[32]. In this study, we standardized and evaluated a qPCR protocol with greater safety that displayed good potential for incorporation, as a complement, into the diagnostic scheme of VL within reference diagnostic centers. Through the monitoring of a greater number of patients (co-infected or not) before, during, and after treatment, the applicability of this technique for the monitoring of parasite load may be established.

In conclusion, the evaluation of the new duplex VL qPCR technique indicated good potential for the accurate, fast, and reliable detection of *L. infantum* DNA, when applied as a complement to the classical diagnostic tools already available and as an alternative for clarifying possible inconclusive cases, especially in health or research reference centers.

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Conflict of interest

The authors declare that they have no conflict of interest.

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