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Multiplex qPCR assay to determine *Leishmania infantum* load in *Lutzomyia longipalpis* sandfly samples

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Abstract

The study aimed to develop a multiplex qPCR to detect *Leishmania infantum* load in different sandfly sample settings using *Leishmania* kDNA and sandfly vacuolar ATPase (VATP) subunit C as internal control gene. The amplification of *Lutzomyia longipalpis* VATP gene was evaluated together with *Leishmania infantum* kDNA in a multiplex reaction. The concentration of VATP gene oligonucleotides was adjusted until no statistically significant difference was observed between all multiplex standard curves and singleplex curves, that is, only kDNA amplification. Limit of detection (LoD) was measured using a probit model and a cut-off defined by receiver operating characteristic analysis. Limit of quantification (LoQ) was assessed by a linear model using the coefficient of variation threshold of 25%. After assuring VATP gene amplification, its primer–probe concentrations were best at 100 nM/10 nM, respectively. The cut-off C_q value for *L. infantum* kDNA was defined as 35.46 with 100% of sensitivity and specificity. A total of 95% LoD was determined to be of 0.162 parasites while LoQ was 5.858. Our VATP/kDNA multiplex qPCR assay shows that it can be used to evaluate both DNA integrity and determine *L. infantum* load in *L. longipalpis* even for low yielded samples, that is, individual midguts.

KEYWORDS
insect control gene, multiplex qPCR, phlebotomine, visceral leishmaniasis

INTRODUCTION

Sandflies are responsible for the transmission of *Leishmania* in the Americas, where the main vector species responsible to transmit the etiologic agent for visceral leishmaniasis (VL), *Leishmania infantum*, is *Lutzomyia longipalpis*. Despite recent VL cases in Santa Catarina State, *L. longipalpis* was not found, thus far (Borges et al., 2017). Other sandfly species such as *Nyssomyia neivai* have been collected in this state and have been detected with *L. infantum* DNA when evaluated by molecular techniques (Saraiva et al., 2009; Grott et al., 2015). In other states in Brazil, other sandfly species such as *L. cruzi*, *Pintomyia fischeri* or *Migonemyia migonei* have been proved or suspected to be susceptible to *L. infantum* infection (Guimarães et al., 2016; Falcão de Oliveira et al., 2017; Galvis-Ovallos et al., 2021). This entomological survey together with *L. infantum* DNA detection is a crucial information for the development of control strategies and detection of new vector species candidates.

Such parasite detection has been classically evaluated by microscopy analysis and culture methods in many studies to detect *L. infantum* promastigotes in sandflies (Rangel et al., 1984; Feliciangeli, Reyes and Limongi, 1988; Carvalho et al., 2008). Nonetheless, in field studies, the difficulty and delay to process a big number of samples, the low *Leishmania* prevalence in sandflies, as well as, the low sensitivity of such methods are factors that limit the parasite detection in its vector (Ashford, Desjeux and Deraadt, 1992). Thus, assays that guarantee a higher sensitivity and specificity such as polymerase chain reaction (PCR) have been applied in studies to detect the presence of *Leishmania* DNA in sandflies (de Pita-Pereira et al., 2008; Marcondes et al., 2009; Soares et al., 2011). Although they do not evaluate vectorial competence, only *Leishmania* DNA detection, such finding is very important for eco-epidemiological and screening for potential new vector species.

qPCR is the most sensitive technique allowing the detection and the parasite load quantification in the insect with much more precision than in situ quantification techniques by optical microscopy (Myskova, Votypka and Volf, 2008). However, it is known that depending on DNA extraction protocol, the DNA's quality and quantity may vary and suffer losses due to incomplete cell lysis and nucleic acid binding to surfaces (Mumy and Findlay, 2004). Due to such issues related to DNA extraction, there are higher chances for false-negative results in molecular tests (Gonçalves-de-Albuquerque et al., 2014). Thus, it is advisable to use control genes to guarantee that the extracted genetic material is intact, with enough amount and without inhibitors (de Pita-Pereira et al., 2008; de Cássia-Pires et al., 2017).

Several genes such as *L. longipalpis'* periodicity (per), cacophony (cac) and vacuolar ATPase subunit C (VATP) have been applied for DNA integrity check in qPCR protocols to identify *Leishmania* DNA in sandflies (Ranasinghe et al., 2008; Bezerra-Vasconcelos et al., 2011; Araujo-Pereira et al., 2020). Both per and cac genes are related to males' mating behaviour and their love songs to attract females (Bauzer et al., 2002; Lins et al., 2002). On the other hand, VATP gene is associated with proton pump in the midgut of sandflies being highly expressed during a blood meal, which highlights its potential as a marker gene for individual midgut and/or female samples (Ramalho-Ortigão et al., 2007).

A multiplex qPCR was developed to detect *L. infantum* kDNA using the mammal 18S gene as a control for the reaction and DNA extraction from dogs with VL (Rampazzo et al., 2017). Thus, it is possible to observe if, according to 18S detection, the DNA in the parasite's kDNA negative samples was intact or not using a smaller amount of DNA sample. Such an approach could be interesting for *L. infantum* kDNA detection in sandfly samples due to its low amount of genetic material after DNA extraction, which contributes to lower resuspension volume in order to achieve higher DNA concentration, leaving a small sample template to be tested (Caligiuri et al., 2019). Moreover, due to its higher sensitivity to detect *Leishmania* in sandfly samples (Galluzzi et al., 2018), kDNA together with a sandfly gene such as VATP, highly expressed in the midgut, constitutes an interesting combination for detection of *L. infantum* in sandfly, especially in individual midguts.

So, the aim of the study was to develop a multiplex qPCR to detect sandfly VATP gene and to simultaneously quantify *Leishmania* kDNA on different sample settings from both wild-caught or colony-reared specimens such as individual and pooled males and females, midguts, with or without a blood meal.

MATERIAL AND METHODS

Primers–probe design

Oligonucleotides were designed for *L. longipalpis* genetic material detection as a control gene to validate the DNA extraction assuring that genomic material was present and with good quality for amplification. The oligos were based on *L. longipalpis* VATP mRNA, under GenBank accession no. EF156436.1. To detect and quantify *L. infantum*, previously described primers and probes (Francino et al., 2006) for the parasite's kDNA were used (Table 1). VATP oligos specificity was tested in silico using BLAST (Basic Local Alignment Search Tool) for each one and for the amplicon.

Sample description

All *Leishmania* negative sandfly samples used for the VATP/kDNA multiplex qPCR standardization were originated from a *L. longipalpis* colony (Laboratório de Interação Parasito-Hospedeiro e Epidemiologia—LaIPHE/Instituto Gonçalo Moniz) ensuring that they were indeed *Leishmania* free since vertical transmission does not occur. Positive samples were obtained either by means of artificial or natural (xenodiagnosis) infection of sandflies and parasite presence has been confirmed by midgut dissection and microscopy observation of promastigotes. All midgut samples were dissected using sterile dissection pins with sterile saline and transferred directly into a 1.5 ml microtube containing 100 μl of Qiagen's tissue lysis buffer ATL and then stored at −20°C until DNA extraction.

TABLE 1 Primers and probes used in the study to detect and quantify both sandfly VATP and *Leishmania infantum* genes

Gene	Oligos	Amplicon size (bp[b])
VATP-F[a]	5′-CGGCTCCTGGCGACAAGACA-3′	143
VATP-R[a]	5′-CGACAGGCCCACGAGCTGAT-3′	143
VATP-P[a]	5′-HEX-CCCGGACCTCAAGGTGGGC ACCC-BHQ1-3′	143
kDNA-F	5′-AACTTTTCTGGTCCTCCGGGT AG-3′	120
kDNA-R	5′-ACCCCCAGTTTCCCGCC-3′	120
kDNA-P	5′-FAM-AAAAATGGGTGCAGAAAT-MGB/NFQ-3′	120

[a]F, R and P stands for forward primer, reverse primer and probe, respectively.
[b]bp, base pairs.

DNA extraction

DNA extraction was performed with DNeasy Blood and Tissue extraction kit (Qiagen) following the manufacturer's manual with some volume adaptations between 1 and 2 weeks after −20°C storage. Briefly, midguts and whole sandflies, female or male, were isolated and ground using autoclaved plastic pestles in a 1.5 ml microtube filled with 100 µl of the kit's tissue lysis buffer ATL and 20 µl of proteinase k (20 mg/ml). Then all tubes were incubated in a water bath at 56°C for at least 3 h and 100 µl of another lysis buffer AL were added followed by 15 s vortex, 100 µl of ethanol, one more 15 s vortex and then centrifuged in the spin column for DNA retention into the membrane. Further washing steps were performed as described by the manufacturer's manual and all samples were eluted with 50 µl of the elution buffer.

L. infantum standard curve

Both singleplex and multiplex standard curves have been mounted using a 200 µl aliquot of 1×10^7/ml cultured *L. infantum*, extracted with the same kit and protocol for sandflies. Ultra-pure water has been used to dilute the singleplex curve in a 10-fold serial dilution to obtain six standard points with equivalent numbers of 10^4, 10^3, 10^2, 10, 1 and 0.1 parasites. For the multiplex standard curve, the same process has been applied but instead of water, the dilution has been made with noninfected female sandfly genomic DNA.

qPCR protocol

Several tests under different concentrations were performed until the establishment of a proof of concept. All reactions have been performed in an ABI7500 Fast Real-Time PCR System (Thermo) using the following cycling protocol: 1 × 95°C/10 min; 45× [95°C/15 s, 60°C/60 s]. Reactions occurred in a volume of 25 µl being 5 µl DNA template and a 20 µl mix of primers, probe and mastermix. For all reactions, 8.34 µl of Multiplex PCR Mastermix (IBMP, Brazil) has been used.

Verification of sandfly VATPase gene amplification

At first, VATP primers were used at 300 nM testing different probe concentrations, 600, 300 and 150 nM to verify gene amplification by this set of primers and probe (Figure S1). For this first reaction individual females with and without blood meal as well as pools of females and males have been used.

Multiplex sensitivity optimization

To assess differences in *Leishmania* detection sensitivity, both multiplex, VATP and kDNA detection; and singleplex, only kDNA detection, reaction protocols have been assayed in the same plate using experimentally infected females. A previously standardized singleplex protocol to detect *L. infantum* kDNA was used (Rampazzo et al., 2017). Thus, primers and probes for VATP and *L. infantum* kDNA were optimized at 100 nM/10 nM and 900 nM/200 nM, respectively. Concentrations for VATP were determined as the lowest without reducing *L. infantum*'s curve C_q values of the multiplex protocol when compared with the singleplex.

Testing different sample situations

To assess C_q values of different sandfly samples for VATP gene, multiplex reactions using primers and probes of VATP and kDNA at 100 nM/10 nM, and 900 nM/200 nM, respectively, have been performed with sandfly samples such as 1) midguts, i - uninfected midgut, ii - infected midgut, iii - pools of 10 uninfected midguts; 2) female sandflies, iv - uninfected and unfed female, v - infected female, vi) pools of 10 uninfected females, vii - blood-fed female 3) and males, viii - males individually and ix - in pools of 10 males. All these nine conditions and sample types were tested with three biological replicates in triplicates.

Cut-off determination, the limit of detection and quantification

After multiplex qPCR protocol standardization, the cut-off was defined by means of receiver operating characteristic (ROC) analysis to obtain the lowest C_q with 100% specificity and the highest sensitivity. For this test, *L. infantum* positive and negative female sandflies have been used. To assess the limit of detection (LoD) and limit of quantification (LoQ), curve-fitting methods have been applied as proposed in the literature (Klymus et al., 2020). Using the ROC curve, the cut-off was defined; LoD was measured by a probit model estimating the number of parasites based on a 95% probability of detection. For such estimation, eight 10-fold dilutions of a sample containing genomic material from a sample with genomic DNA of about 100 *L. infantum* promastigotes have been used. Eight replicates were used per serial dilution for this reaction except for the two lowest concentrations, that is, 10^{-4} and 10^{-5}, which were represented by 12 replicates. LoD was calculated based on $LoD = \frac{N-Pi}{S}$, being N the number of parasites with 95% of detection according to the model, Pi the probit's interceptor and S the model's slope. LoQ was also assessed using the results of the same reaction as for LoD, fitting a linear model with the coefficient of variation (CV) of each tested concentration, which was used to predict the CV of *Leishmania* concentrations ranging from 0.001 to 1000 parasites, increasing by 0.001. Then, LoQ has been defined as the lowest concentration with a CV < 25% according to the model as previously proposed and defined in the literature (Klymus et al., 2020).

Statistical analysis

In order to, identify the multiplex concentration protocol with no differences in *L. infantum* standard curve C_q values in comparison with

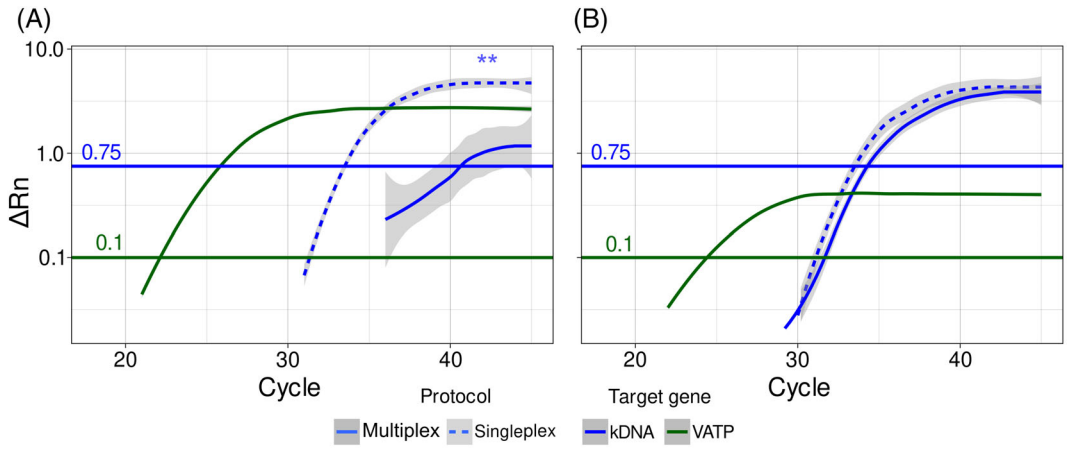

FIGURE 1 Singleplex and multiplex comparison using VATP primer–probe concentration at 300 nM/150 nM from a 0.1 *L. infantum* standard curve (a). On B, another singleplex and multiplex comparison is demonstrated using VATP primer–probe at 100 nM/10 nM of a 0.1 *L. infantum* standard curve. The modelled curves using a loess regression of each curve triplicates' ΔRn are plotted with the standard error projected in grey. ** represents a $p < 0.01$ for the ad hoc Tukey test

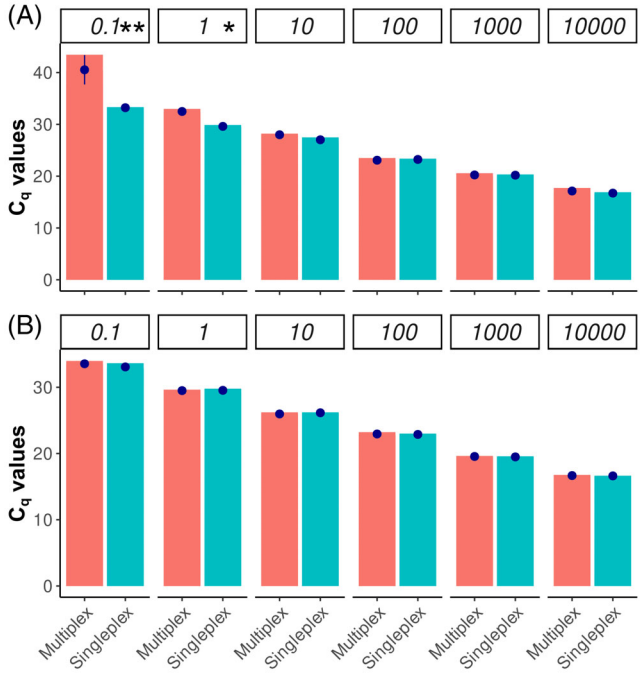

FIGURE 2 Bar plot of the comparison between multiplex and singleplex standard curves when primer–probe was used at 300 nM/150 nM (a) and 100 nM/10 nM (b). * for $p < 0.05$ on Tukey's post hoc test, ** for $p < 0.01$ on Tukey's post hoc test. Blue dots and lines represent the mean C_q values and their minimum and maximum amplitude

the singleplex, a one-way ANOVA followed by Tukey's HSD (Honest Significant Difference) method have been applied to compare all standard curve points from the singleplex with all multiplex concentration protocols (Figure S2). All analyses have been performed in R v4.0.2 using the stats package for the regression models, that is, probit and linear regression of standard curves, one-way ANOVA (Analysis of Variance) and Tukey's post hoc tests. ROC analysis was performed with the pROC package (Robin et al., 2011). For all analyses, statistically significant differences were considered for $p < 0.05$. Curve efficiency was measured as $-1 + 10^{(-1/slope)}$.

RESULTS

VATPase gene amplification

Figure S1 shows results using primers at 300 nM and probe at 150 nM of one unfed female sandfly sample under 10-fold serial dilution until 0.001 individual sandfly dilution, demonstrating that even a small portion of sandfly genetic material would be amplified. In an in silico evaluation of VATP's amplicon specificity, only *L. longipalpis* VATPase subunit C mRNA hits have been found with 100% identity and query coverage with an e-value of 7e-67. When the forward primer was evaluated in an isolated way, no other arthropod showed 100% identity. For the reverse primer and probe, there was 100% identity with *Drosophila sp*. predicted mRNA sequences but with high e-values, >3.

Multiplex reaction standardization and sensitivity refinement

After observing a higher C_q value for some concentrations of the multiplex curve in comparison with the singleplex curve (Figure 1a), lower concentrations of VATP primers and probe have been tested in order to find the lowest concentration with minimum or no sensitivity loss. Figure 1b shows the results of 100 nM/10 nM primer–probe concentration, which presented no differences in C_q values when compared with the singleplex curve of the same dilution. Due to its lower concentration, the curve was also lower, and therefore, the threshold for VATP gene has been lowered to 0.1 corresponding to its new exponential phase.

When primer–probe was used at 100 nM/10 nM no statistically significant difference (ANOVA and ad hoc Tukey tests) was present for all multiplex standard curves in comparison with singleplex curves. In Figure 2a, the results of such comparisons are shown from the highest VATP primer–probe concentrations, that is, 300 nM/150 nM, showing statistically significant differences on the lowest curve points. The results of the above-mentioned singleplex–multiplex comparison using 100 nM/10 nM VATP primer–probe concentrations without statistically significant differences are displayed in Figure 2b.

TABLE 2 Mean and standard deviation of VATP C_q values from different sandfly samples under different variables such as presence of infection, blood meal, number of sandflies, gender and isolated midgut tissue conditions and kDNA standard curves. The intra-assay variation is also presented as the coefficient of variation from all replicates of each condition (9 replicates) or standard curve (12 replicates)

VATP—sample conditions	VATP mean C_q (SD[a])	Intra-assay variation (%CV[b])
Individual uninfected and unfed female	22.68 (± 0.21)	14.78
Individual infected female	22.76 (± 0.60)	43.57
Individual blood-fed female	23.16 (± 0.30)	21.13
Pool of 10 uninfected and unfed females	20.10 (± 0.20)	13.90
Individual uninfected midgut	24.59 (± 0.38)	26.46
Individual infected midgut	24.99 (± 0.36)	25.24
Pool of 10 uninfected midguts	22.01 (± 0.23)	16.13
Individual male	24.32 (± 0.36)	25.66
Pool of 10 males	21.03 (± 0.16)	10.78
kDNA—multiplex standard curves	kDNA Mean C_q (SD)	
10^4	15.38 (± 0.25)	17.31
10^3	18.31 (± 0.26)	17.97
10^2	21.63 (± 0.65)	47.57
10	25.19 (± 0.77)	57.80
1	29.02 (± 0.75)	55.42
0.1	33.18 (± 0.72)	53.18

[a]SD—Standard deviation.
[b]CV—Coefficient of variation.

Different sample situations

There was VATP amplification for all tested sample conditions, even for an individual midgut. The VATP C_q mean values varied between 20.1 and 24.99 throughout all sample types and variables such as the presence of infection, blood meal, number of sandflies, gender and tissue conditions (Table 2). Mean VATP C_q values were 22.68 for individual female sandfly without infection and blood meal, 22.76 for individual female sandfly with *L. infantum* infection, 23.16 for individual female sandfly with blood meal and 20.1 for pools of 10 uninfected female sandflies. For the midguts, the mean C_q values were 24.59 for individual uninfected midgut, 24.99 for infected midgut and 22.01 for

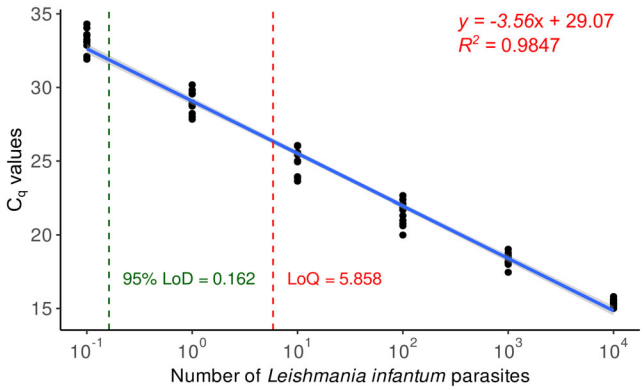

FIGURE 4 Linear regression of 10-fold serial dilution of the *L. infantum* standard curve with 12 replicates containing 0.1 to 10,000 promastigotes. The grey shadow beneath the blue line represents the 95% confidence interval, while black dots represent each replicate. Green and red dashed lines represent the limit of detection (LoD) and the limit of quantification (LoQ), respectively

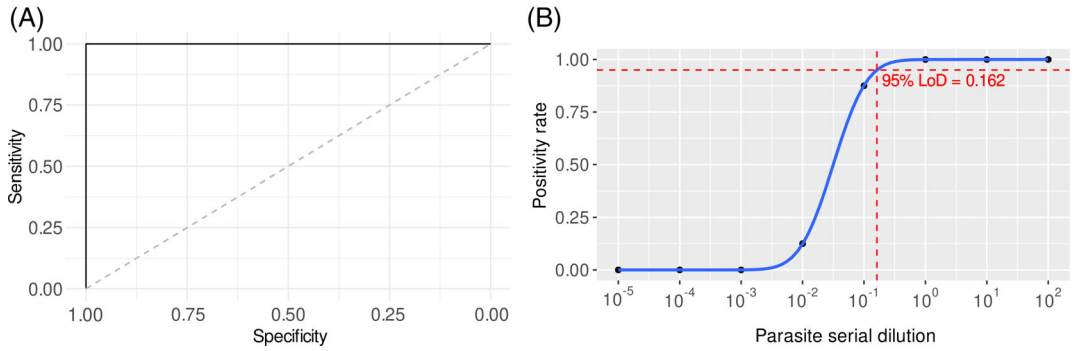

FIGURE 3 Receiver operating characteristic analysis for cut-off estimation (a) and 95% limit of detection (LoD) assessed by probit linear model (b)

pools of 10 uninfected midguts. As for male sandflies, it was 24.32 for individual male and 21.03 for pools of 10 males.

Analytical sensitivity assessment

Based on the ROC curve analysis, the C_q of 35.46 was defined as the cut-off for *Leishmania* DNA detection, whereby both sensitivity and specificity were 100% (Figure 3a). The LoD was measured by a probit model approach, which resulted in 0.162 *Leishmania* parasites (Figure 3b).

The VATP/kDNA multiplex qPCR's LoQ has been calculated reflecting the quantification of 5.858 *L. infantum* parasites as the minimum quantifiable parasite load with a 25% CV. As for the standard curve's regression using 12 replicates of each curve point, the intercept and slope have been estimated to be 29.07 and −3.56, respectively, with R^2 of 0.98 and efficiency of 90.94%, shown in Figure 4. For all reactions, NTCs presented no amplification for both genes.

DISCUSSION

Molecular diagnostic techniques have brought more sensitivity and specificity to *Leishmania* detection in sandflies in face of classical methods such as in situ observation of promastigotes in dissected midguts (Rêgo et al., 2015). However, despite its high sensitivity and specificity, the DNA extraction quality should be assessed to reduce false-negative results due to the presence of inhibitors and low DNA integrity in the sample (Arif et al., 2021). Thus, we have developed a multiplex qPCR assay that detects both the sandfly VATP gene as an internal control and *L. infantum* kDNA to quantify the parasite load in female sandflies as well as in individually dissected midguts.

Our protocol was able to detect sandfly DNA even in a single female sample 1000 times diluted or a single midgut sample. None of the qPCR assay standardizations present in the literature have evaluated small *L. longipalpis* samples such as an individual midgut (Ranasinghe et al., 2008; Bezerra-Vasconcelos et al., 2011; Cunha et al., 2014). Several studies detecting *Leishmania* parasites in sandfly samples have used an extra reaction promoting the usage of more sample material, which is scarce in cases where DNA has been extracted from only one sandfly (Rodrigues et al., 2016; de Sousa Ferreira et al., 2018). For a multiplex reaction approach, some studies have used sandfly constitutive genes such as cacophony, periodicity and also VATP (Rodrigues et al., 2014; Araujo-Pereira et al., 2020). Although the comparison between these genes is not possible due to differences in both extraction and qPCR protocols, all of them have been successfully used as an internal control gene to certify DNA integrity. In the present work, it has been demonstrated that VATP gene can also be used for such evaluation even for low yielded samples, that is, individual midgut.

Little is known about VATP copy numbers in female or male sandflies. Our results showed higher C_q values in male samples when compared with females both for pooled and individual samples, suggesting that there are more copies of VATPase subunit C gene in female sandflies. Thus, when used as a reference for quantification in multiplex qPCR assays with SYBR Green as in Bezerra-Vasconcelos et al. (2011), these differences in copy number among sample types should be taken into account in cases where standard curves are built using males.

The use of an individual sandfly sample for *Leishmania* detection brings us much more precise and closer information regarding the true infection rate in a sandfly population. However, due to financial and logistic reasons, the infection rate of a population has been assessed by using pooled samples and calculating a minimum infection rate (MIR) (Bustamante and Lord, 2010). Such an approach has lower costs and is less time-consuming, which is advantageous for a large-scale epidemiological study. On the other hand, MIR is an estimation of the infection rate and its precision is affected by sample size, the number of pools, true infection rates and the number of individuals per pool (Gu, Lampman and Novak, 2003). In xenodiagnosis and experimental infection studies, the parasite load information is much more important to compare hosts' infectiousness or different infection conditions (Borja et al., 2016). Therefore, for such studies evaluating lower sandfly numbers, assessing the parasitemia individually is more feasible avoiding loss of information on *Leishmania*–invertebrate host relationship that would be mixed and diluted in pooled samples.

Using the multiplex VATP/kDNA qPCR herein standardized, the LoD was 0.162 *L. infantum* parasites per reaction. When compared with other multiplex qPCR assays to detect *Leishmania* kDNA in sandfly samples, a lower LoD, 0.004 *Leishmania* parasite–reaction, has been observed by Bezerra-Vasconcelos et al. (2011). However, an SYBR green-based qPCR was applied and the methods to determine LoD were not precisely described without information regarding a previous C_q cut-off value to determine, which curve replicates would be considered positive or negative. Therefore, their estimation could be assuming false-positive results due to SYBR Green binding to non-specific DNA fragments and negative samples in LoD calculation due to a lacking cut-off value, reflecting only the last tested curve point. Based on the detection–non-detection characteristic of LoD's calculation (Stokdyk et al., 2016), a previous cut-off determination allows a more precise LoD in terms of qPCR efficiency.

For the LoQ, the lowest concentration with a CV < 25% was 5.858 *L. infantum* parasites/reaction. Although LoQ is an important parameter to evaluate the minimum amount of parasite with a stated precision, assessed by CV (Klymus et al., 2020), none of the studies standardizing qPCR assays to detect *Leishmania* kDNA have demonstrated their LoQ. Nonetheless, our LoQ of 5.858 *L. infantum* per reaction highlights the number of parasites to be considered quantifiable by our VATP/kDNA multiplex qPCR, meaning that samples with C_q higher than the cut-off and lower quantification than the LoQ should be only qualitatively considered.

Despite its high sensitivity and specificity, qPCR assays do not distinguish live and active parasites from dead parasites as well as the infective metacyclic promastigote proportion in the sandfly midgut. Although laborious, such important information can only be evaluated by means of midgut dissection followed by in situ observation by an optical microscope (Borja et al., 2016). However, due to its low

sensitivity leading to higher false-negative rates, it is not advisable to perform only in situ observation of promastigotes to evaluate and detect *Leishmania* infection in sandflies (González et al., 2017). Thus, after dissection and microscopic observation, it is highly advisable to certify the *Leishmania* negative midguts with a molecular technique such as qPCR, which can more precisely determine parasite load (Myskova, Votypka and Volf, 2008). This approach can be achieved by our qPCR assay, which, at the same time, detects and quantifies the parasite's kDNA as well as assures the sample's DNA integrity for sandfly midgut samples.

The VATP/kDNA multiplex qPCR herein standardized can be used to evaluate both DNA integrity and determine *L. infantum* parasite load in *L. longipalpis* even for low yielded samples such as individual midgut. This multiplex approach will aid researchers to determine the parasite load transmitted to sandflies in future xenodiagnostic studies as also the rate of *L. infantum* positive *L. longipalpis* of a population for small-scale entomological surveys.

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AUTHOR CONTRIBUTIONS

Tiago Feitosa Mota: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization; Claudia Ida Brodskyn: Resources, Writing - Review & Editing, Funding acquisition; Luis Gustavo Morello: Writing - Review & Editing, Resources; Fabricio Klerynton Marchini: Writing - Review & Editing, Resources; Marco Aurelio Krieger: Writing - Review & Editing, Resources; Rita de Cássia Pontello Rampazzo: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision; Deborah Bittencourt Mothé Fraga: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration.

CONFLICT OF INTEREST

None of the authors have conflicts of interest to declare.

ETHICS STATEMENT

For the blood-fed female sandflies all artificial feeding and xenodiasgnostic experiments were ethically approved by Intituto Gonçalo Moniz's committee for animal experimentation [CEUA 014/2016].

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Singleplex assay using VATP primer/probe concentration at 300 nM/150 nM from one unfed female sand fly sample under 10-fold serial dilution until 0.001 individual sand fly dilution. The modelled curves using a Loess regression of each curve triplicates' ΔRn are plotted with the standard error projected in grey.

Figure S2. Bar plot of the comparison between duplex and singleplex standard curves when primer/probe were used at 300 nM/150 nM (A), 150 nM/75 nM (B), 150 nM/37.5 nM (C), 100 nM/20 nM (D) and 100 nM/10 nM (E). * for $p < 0.05$ on Tukey's post-hoc test, ** for $p < 0.01$ on Tukey's post-hoc test. Blue dots and lines represent the mean C_q values and its minimum and maximum amplitude.

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