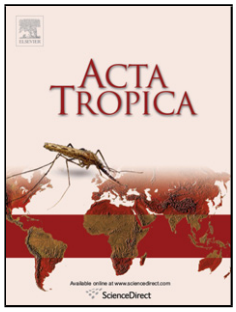

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The applicability of real-time PCR in the diagnostic of cutaneous leishmaniasis and parasite quantification for clinical management: current status and perspectives

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Abstract

Cutaneous leishmaniasis (CL) is spread worldwide and is the most common manifestation of leishmaniasis. Diagnosis is performed by combining clinical and epidemiological features, and through the detection of *Leishmania* parasites (or DNA) in tissue specimens or trough parasite isolation in culture medium. Diagnosis of CL is challenging, reflecting the pleomorphic clinical manifestations of this disease. Skin lesions vary in severity, clinical appearance, and duration, and in some cases, they can be indistinguishable from lesions related to other diseases. Over the past few decades, PCR-based methods, including real-time PCR assays, have been developed for *Leishmania* detection, quantification and species identification, and improving the molecular diagnosis of CL. This review provides an overview of many real-time PCR methods reported for the diagnostic evaluation of CL and some recommendations for the application of these methods for quantification purposes for clinical management and epidemiological studies. Furthermore, the use of real-time PCR for *Leishmania* species identification is also presented. The advantages of real-time PCR protocols are numerous, including increased sensitivity and specificity and simpler standardization of diagnostic

procedures. However, despite the numerous assays described, there is still no consensus regarding the methods employed. The analytical and clinical validation of CL molecular diagnosis has not followed international guidelines. A consensus methodology comprising a DNA extraction protocol with an exogenous quality control as an internal reference to normalize parasite load is still needed. In addition, the analytical and clinical performance of any consensus methodology must be accurately assessed. This review shows that a standardization initiative is essential to guide researchers and clinical laboratories towards the achievement of a robust and reproducible methodology, which will permit further evaluation of parasite load as a surrogate marker of prognosis and monitoring of aetiological treatment, particularly in multi-centric observational studies and clinical trials.

Introduction

Leishmaniasis is a protozoan vector-borne disease caused by a parasite from the genus *Leishmania* (Trypanosomatida: Trypanosomatidae). Leishmaniasis is considered the most neglected tropical disease, according to the disability adjusted life years (DALYs) (Hotez et al, 2015). Globally, approximately 12 million people are infected, and 350 million individuals live in risk areas (Alvar et al, 2012). After parasite inoculation, the infection outcome ranges from asymptomatic to disease symptoms. The disease presents different manifestations, including its three most common forms: visceral leishmaniasis (VL), mucosal leishmaniasis (ML) and cutaneous leishmaniasis (CL). Asymptomatic infection is the most common outcome after parasite inoculation and can contribute to the transmission of this disease (Andrade-Narvaez et al 2016).

CL is the most common manifestation of leishmaniasis worldwide. Clinical symptoms range from small-localized lesions to disseminated large ulcers all over the body. The overall prevalence of leishmaniasis is approximately 1.2 million people per year (Alvar et al., 2012). The most prevalent species causing CL are *Leishmania (Leishmania) tropica and L. (L.) major* in the Eastern Hemisphere *and L. (L.) mexicana, L. (Viannia) braziliensis,*

L. (V.) guyanensis, and L. (V.) panamensis in the Western Hemisphere (WHO 2010).

The clinical spectrum of CL is variable and can be misdiagnosed as other skin conditions, but clinical and epidemiological criteria are still essential for diagnosis. However, high-specificity parasitological diagnosis remains essential. *Leishmania* parasites in tissue specimens are detected using microscopic examination, cultivation and/or molecular techniques (WHO 2010). Molecular approaches offer high potential for direct application in clinical samples, avoiding the isolation and cultivation of the parasites, and enabling parasite quantification.

In recent years, real-time PCR assays for *Leishmania* detection, quantification and species identification have improved the molecular diagnostic analysis of CL. Nevertheless, the higher analytical sensitivity compared to conventional PCR is arguable (Bastien, 2008), and the number of real-time PCR assays reported in the literature increases every year, highlighting the advantages of the technique, as the speed to obtain results, the broad dynamic range to quantify DNA targets and reduction in cross-contamination since there is no need for handling the PCR products to perform gel electrophoresis.

Herein we discuss the benefits of real-time PCR to diagnose and estimate parasite load in skin lesions from patients with cutaneous leishmaniasis and concomitant species or subgenus discrimination through melting or high-resolution melting analysis. We also present evidence on the potential of parasite load as a biomarker for treatment outcome and disease prognosis. Finally, we address the need for conceptual definitions and the establishment of consensus protocols, showing that several methodologies were tested without consensus regarding protocols and molecular targets, impairing data comparison.

Targets

Leishmania parasites present a genome varying from 29 to 33 Mb in size, organized in a variable number of chromosomes, ranging from 34 to 36 (Cantacessi et al., 2015, Real et al., 2013). Despite biological differences among *Leishmania* species, including their association with different clinical

manifestations, there is high similarity among their genome, with an elevated degree of conservation in gene content and architecture (Peacock et al, 2007; Real et al., 2012; Cantacessi et al., 2015). *Leishmania*, similar to other trypanosomatids, possesses a single mitochondria organelle, the kinetoplast, which contains DNA (kDNA) organized in a large network of concatenated minicircle (approximately 1 Kb each, with $5x10^3$ to $5x10^4$ per parasite) and maxicircle (approximately 23 Kb each, with 20 to 50 per parasite) molecules (Simpson et al., 1986; Lukeš et al. 2002).

So far, several molecular targets for *Leishmania* molecular diagnosis have been described for conventional and real-time PCR assays, but kDNA has traditionally been the most reported target, reflecting its elevated number of minicircle copies per parasite genome and the distinctive presence of the kinetoplast in trypanosomatids, which yields high sensitivity and specificity for molecular detection of the parasite in diverse clinical samples collectable in distinct forms of leishmaniasis (Lopez et al, 1993, Mary et al., 2004, Wheirather et al., 2011, Pita-Pereira et al., 2012, Jara et al., 2013). However, minicircle heterogeneity and copy number variation among *Leishmania* species and strains might affect the accuracy of assays using kDNA (Mary et al 2004, Weirather et al 2011). Apart from kDNA, different regions of ribosomal RNA genes (rRNA) have also frequently been used. The 18S rRNA region, part of the eukaryotic ribosome small subunit (SSU), is commonly utilized for the diagnosis of different *Leishmania* species, reflecting its highly conserved nature, whereas Internal transcribed spacer (ITS) regions are used for species-typing, reflecting high-variability among species (Schönian et al. 2001; Kuhls et al. 2005). With respect to the 18S rRNA region, studies have shown that RNA amplification methods are more sensitive than conventional PCR (van der Meide et al 2008), as RNA amplification targets rRNA copies, which are more abundant than ribosomal DNA (Van Eys et al 1992; van der Meide et al 2005). However, a disadvantage is the high prices for this molecular assay. In addition, some conventional and real-time PCR assays targeting protein coding genes in *Leishmania*, such as Heat Shock Protein with 70 KDa (HSP70) (Graça et al. 2012, Zampieri et al 2016), Catalytic subunit of DNA polymerase A (Weirather et al., 2011), Glucose-6-phosphate dehydrogenase (G6PD, Castilho et al., 2008, Jara et al, 2013), etc., have also been reported. In general, these

alternative targets present high specificity to detect *Leishmania* species, but less sensitivity compared to targets with elevated copy numbers, such as kDNA minicircle molecules. *Leishmania* targets reported in real-time PCR assays for the diagnosis of leishmaniasis, parasite load quantification and/or parasite species discrimination are shown in the supplementary material (Table S1).

Table 1 summarizes the characteristics of 28 non-redundant real-time PCR studies, published from 1993 to 2017, targeting *Leishmania* kDNA, SSUrRNA, HSP70 and other molecules. In March 2017, when the terms “real-time PCR cutaneous leishmaniasis” were used as query in PubMed (https://www.ncbi.nlm.nih.gov/pubmed), over 140 manuscripts are indicated as search results. Nevertheless, most of these methods apply similar assays as those described. Considering the studies reported in Table 1, 12 studies (42.9%) employ *Leishmania* kDNA minicircles as molecular targets, 11 studies (39.3%) employ ribosomal RNA genes (SSUrRNA or ITS) and 3 studies (10.7%) employ HSP70. The use of kDNA and ribosomal RNA genes as molecular targets for the diagnosis of leishmaniasis in most of the studies likely reflects the high sensitivity of these assays. Regarding the dye/fluorophore, SYBR Green and TaqMan were used in 23 and 19 assays, respectively; 29 reactions were developed to estimate the parasite load and 11 reactions were developed only to detect *Leishmania*. Other dyes/fluorophores, such as MeltDoctor and LightCycler probes, were reported in 8 assays. Most of these studies reported the use of an internal reference, generally a human target, such as β-actin, RNAse P, etc., to monitor PCR inhibition and false-negative results. However, only a few studies described the normalization of parasite load to the amount of human DNA, which is an important issue when DNA is extracted from solid samples, such as skin tissue. In addition, 26 reactions could simultaneously detect and differentiate *Leishmania* species by normal and high-resolution melting curves, TaqMan or LightCycler probes. Table S1 reports the detailed characteristics of each assay, including primer and probe sequences and references.

Gomes et al (2017) compared the accuracy of qPCR using SYBR Green and TaqMan for the diagnosis of CL and ML from swabs and biopsy samples using two sets of primers/probe targeting *L. (Viannia)* kDNA, reaching 100%

specificity for both methodologies and sample types. However, sensitivity varied by sample type and method: for biopsy samples, sensitivity (95% CI) was 84.72% (74.68–91.25) using SYBR Green versus 73.61% (62.42–82.41) using TaqMan; and for swab samples, sensitivity was 87.88% (77.86–93.73) using SYBR Green versus 78.79% (67.49–86.92) using TaqMan. Although the assay using SYBR Green presented higher sensitivity for both types of samples, this result should be carefully considered since two different sets of primer/probes were used. The results could be somewhat biased by the efficiency of PCR in each assay. In the mentioned publication, the results did not indicate that SYBR Green is superior to TaqMan, as the observed higher SYBR Green sensitivity might reflect differences in DNA sequences at primer annealing sites for the *Leishmania* strains analysed, rather than the chemistry itself.

In terms of costs, TaqMan assays are typically more expensive. However, due to the potential to perform multiplex reactions, which is not possible using SYBR Green, the final cost of TaqMan assays could be lower. The addition of an internal reference human gene as a target in multiplex assays is crucial to assure the quality of the DNA sample, particularly to avoid false-negative results and assess the presence of potential PCR inhibitors and potential loss of DNA during extraction. Another advantage relies on the specificity gain that TaqMan probes add to the reaction. However, SYBR green-based assays and PCR with high-resolution melting permit detection, followed by the discrimination of *Leishmania* subgenus or species, according to the melting curve generated for each sample in the assay. Table 1 shows that 7 studies (28%) reported the use of SYBR Green assays to genotype *Leishmania* by melting curve, and 6 studies applied TaqMan or LightCycler probes to discriminate parasite species. Additionally, 15 out of 25 studies (60%) reported the use of internal reference to monitor the presence of false-negative results or the normalization of parasite load by human DNA content. In the absence of normalization by DNA contents, there may be a bias in the comparison of parasite load between different samples and studies.

Sample collection

According to the clinical practice guidelines of the Infectious Diseases Society of America and the American Society of Tropical Medicine and Hygiene for the diagnosis and treatment of Leishmaniasis (Aronson et al., 2016), samples for diagnosing CL should be collected from an active (vs. a nearly healed) skin lesion. Frequently used methodologies for harvesting samples include scraping or brushing the debrided ulcer base or edges, aspirating lesions, and obtaining skin snips or punch/shave biopsy specimens from an indurated border.

There are controversies concerning the influence of sampling methods on the sensitivity of diagnostic methods to CL. Furthermore, whether sensitivity could also be affected by the number and distribution of parasites in lesions associated with its chronicity is also debatable (Suárez et al 2015, Herwaldt 1999, Robinson et al 2002, Ramirez et al 2000, Weigle et al 2002, Vergel et al 2006). In a comparative study of different skin lesion sampling techniques for diagnosis and species identification in CL (Valencia et al., 2012), the authors demonstrated that cytology brushes offer comparable diagnostic sensitivity and specificity as filter paper lesion impression and scraping when monitored using PCR targeting kDNA. The sensitivity was greater than 98% and the specificity was 100% for all cited methodologies (except for CerviSoft® cytology brush). Nevertheless, cytology brushes offer an advantage over scraping as a completely non-invasive harvesting method.

When real-time PCR targeting kDNA was used to compare parasite load between skin lesion sample harvesting by biopsy, scraping and cytology brushes (Suárez et al, 2015) in patients from Peru, a higher parasite load could be quantified from cytology brushes and scraping compared to biopsies. Measurements on biopsies vs. scrapings or cytology brushes were highly correlated in all lesion sites, indicating that parasite load was consistent through sampling methods, in contrast to other studies, as mentioned above. In the same study, the parasite load was compared among ulcer sites (ulcer base, centre and border). Median parasite load (PL) differed among 3 ulcer sites in biopsies and scrapings, with specimens from the ulcer base and centre presenting significantly higher PL than those from the ulcer border. Indeed, the method should be considered during sample harvesting to increase the sensitivity of diagnosis. In a previous study involving patients from Colombia

(Adams et al., 2014), swab and aspirate samples from 105 lesions of CL suspected patients were evaluated using qPCR targeting 18S rDNA, comparing parasite load between three different DNA extraction protocols to both harvesting methods. The authors observed that less parasite DNA was recovered from lesion aspirates compared to swab samples, regardless of DNA extraction method used, demonstrating the feasibility and diagnostic accuracy of swabbing as a non-invasive alternative to aspirates and biopsies.

Leishmania nucleic acid quantification for monitoring treatment outcome and clinical cure

Parasites persistence in scars can occur after clinical cure of CL or ML. Parasites were detected also in the healthy tissues of patients with active CL (Schubach et al, 1998a and 1998b; Coutinho et al, 2002; Mendonça et al, 2004; Romero et al, 2010; Vergel et al 2006). In some studies, parasites were detected based on the presence of DNA, and viability was not demonstrated based on the detection of RNA molecules. However, the detection of Leishmania gene transcripts corroborates DNA findings (Romero et al 2010; Martínez-Valencia et al 2017), and it was previously demonstrated that DNA molecules are rapidly degraded after parasite death (Prina et al 2007). Evidence of Leishmania persistence was demonstrated using real-time PCR targeting both kDNA and 7SLRNA transcripts, showing 60% of patients presenting parasites at the end of treatment and 30% of patients presenting at 13 weeks follow-up (Martínez-Valencia et al 2017).

The clinical significance of parasite load monitoring, as a biomarker of treatment outcome in leishmaniasis, has been investigated (Mary et al, 2006; van der Meide et al., 2008; Jara et al., 2016; Pereira et al 2016). Although low in number, these studies provide evidence of the applicability of parasite load quantification using real-time PCR for the evaluation of the aetiological treatment efficacy and prognosis. In a study with 44 patients presenting ML (Jara et al., 2016), parasite load was evaluated using qPCR over a period of time (at 14 and 21–28 days of therapy and 3, 6, 12–18, and 18–24 months after treatment) as a potential biomarker of treatment outcome, and two distinct patterns were observed: one pattern was characterized by sequential decline

in parasite load, during and after therapy, until the qPCR was negative, and the other pattern was characterized by a negative qPCR during treatment, followed by an increase in parasite load thereafter. Interestingly, all patients who failed treatment presented the first pattern, exhibiting negative qPCR only after the treatment. It was hypothesized that the parasite persistence may reflect limited access to the drugs, as these parasites are in a quiescent stage or localized in other tissues, distant from the mucosal lesion (e.g., skin). Thus, parasite load rebounding could occur after drug clearance, causing treatment failure. The association of leishmaniasis with multiple infection genotypes has previously been described, resulting in a more aggressive manifestation of this disease, a silent persistence of the parasites or infection associated with parasites less sensitive to the drugs (Brito et al, 2009). Another study (Pereira et al, 2016) showed that parasite loads from primary good and poor responders were indistinguishable but significantly reduced in the first episode of recurrence, indicating that the host has some control over parasite growth. Interestingly, this study demonstrated that *L. braziliensis*-infected patients presenting poor responses to treatment showed lower tissue parasitism than those with a good response.

However, solid evidence concerning the benefit of PCR for monitoring VL treatment have been described, showing high consistency associated with the fact that the PCR from blood samples remains positive after treatment in individuals with reactivation and negative in individuals who evolved to clinical cure (Nuzum et al., 1995; Disch et al., 2004; Maurya et al, 2005). In a prospective cohort study of patients from Spain coinfected with human immunodeficiency virus (HIV) and VL (Molina et al, 2013), the parasite load was monitored using qPCR targeting *Leishmania* kDNA, at baseline and every 3 months until 12 months after initial treatment. Thirty-seven episodes were analysed, and 25 episodes were considered as relapses. A significant decrease in parasite load was observed at 3 months after treatment, which could predict the clinical evolution of VL.

Parasite load, disease pathogenesis and asymptomatic infection

In American cutaneous leishmaniasis, histopathological or molecular-based studies reported differences between the parasite loads in cutaneous or

mucosal lesions (Azogue, 1983; Guttierrez et al, 1991, Jara et al., 2013; Pereira et al 2016). These studies suggest that patients presenting ML show lower parasite load in lesions than patients with CL, which can interfere with the sensitivity of the diagnostic test to detect *Leishmania*. In a cohort from Peru, Jara et al (2013) applied a SYBR green-based qPCR assay targeting kDNA to simultaneously detect and quantify *Leishmania (Viannia)* parasites with high sensitivity in skin and mucosal lesion biopsy specimens. These authors observed a 10 times higher median parasite load in CL lesions (1.0×10^3 parasites/ 10^6 human cells) than in ML lesions (0.9×10^2 parasites/ 10^6 human cells). Similar results were obtained in a study employing the SSU rRNA gene, conducted with 126 patients from Rio de Janeiro, Brazil, all infected with *L. braziliensis* (Pereira et al 2016). A high parasite load (above 10^4 parasites / 10^6 human cells) was observed in the lesions of recent onset (median lesion duration of 64 days), whereas a low parasite load (up to 10^4 parasites / 10^6 human cells) was observed in higher evolution time lesions (median lesion duration of 95 days). Additionally, these authors did not observe a significant correlation between parasite load and patient age, number of lesions, and the mean total area of active lesions or infecting species. In a previous study (Romero et al, 2001), the clinical patterns and diagnostic methods of CL patients infected with *L. braziliensis* or *L. guyanensis,* from two different regions from Brazil (states of Bahia and Amazonas, respectively), were compared. In this study, the clinical findings differed in number, size and location of skin lesions, and characteristics of the lymphatic involvement. Patients infected with *L. guyanensis* presented smaller and more numerous lesions, frequently located above the waist. The lymphatic involvement, evaluated based on the existence of adenomegaly or lymphangitis, was frequently reported for patients infected with *L. braziliensis*. Although parasite load was not evaluated in this study, the detection of *Leishmania* in imprint preparations and skin lesion cultures was more sensitive in patients infected with *L. guyanensis.*

Asymptomatic infections by *Leishmania* spp. comprise an important proportion of the naturally exposed population in endemic leishmaniasis foci (Weigle et al, 1993; Singh et al, 2014; Davis et al, 1995). This phenomenon can be observed in individuals who never experienced leishmaniasis and after treatment and clinical resolution of the disease (Vergel et al, 2006; de Oliveira

Camera et al, 2006). The epidemiological impact of asymptomatic infection in transmission of leishmaniasis is not well known, but it was previously demonstrated that sand flies acquire *L. infantum* from asymptomatic dogs from VL transmission areas (Molina et al 1994; Michalsky et al 2007; Laurenti et al 2013). The evaluation of infected people in endemic foci was primarily based on immunological analysis (e.g., delayed type hypersensitivity response to *Leishmania* antigen or *in vitro* expansion of memory T cells), which cannot distinguish between exposition to the parasite and persistent infection. Few studies present the parasitological demonstration of asymptomatic infection in the context of endemic exposure to the transmission of cutaneous leishmaniasis, although parasite evidence and quantitative data on subclinical infection may contribute to the current understanding of the complex epidemiology of CL. Rosales-Chilama et al (2015) amplified 7slRNA and detected parasites in an important proportion of *Leishmania* Skin Test positives (LST+) individuals and in a small proportion of LST- residents of endemic foci of *L*. (*Viannia*) transmission, demonstrating the technical feasibility of the molecular detection of *Leishmania* in mucosal and blood monocyte samples from individuals with subclinical infections.

Real-time PCR for *Leishmania* species identification

Identification of *Leishmania* species using molecular methodologies is particularly useful in sympatric regions for different pathogens, in areas without a well-known epidemiology, with a high-level of equivocal serological results or presenting a complex travel history or influx of people (Marfurt et al., 2003; Foulet et al., 2007; Gomes et al., 2007; Schijman et al., 2016). Methods for DNA detection using real-time PCR are classified into two main groups, according to the fluorescent agent used and the specificity of the reaction (Navarro et al, 2015): double-strand DNA intercalating agents, such as SYBR Green I, or fluorophores attached to oligonucleotides (probes) that only detect specific PCR amplification products, such as TaqMan or LightCycler probes.

When SYBR Green binds to the minor groove of the dsDNA, its fluorescence is increased and measured during the extension phase of each real-time PCR cycle (Wittwer at al., 1997, Morrison et al., 1998). Reflecting the potential of nonspecific products and primer-dimer formation, a post-PCR

melting curve analysis is necessary to verify the specificity of the reaction. In fact, PCR products of different lengths and GC contents show different peaks at derivative melting curves, corresponding to different melting temperatures (Tm) (Ririe et al, 1997). The property of SYBR Green PCR product differentiation according to melting curve analysis was extensively explored for the molecular typing of pathogens, such as *Cryptosporidium parvum* (Tanriverdi et al, 2002), *Giardia duodenalis* (Amar et al, 2003), *hepatitis B and C virus* (Payungporn et al, 2004; Fugigaki et al, 2004, Zaghloul et al, 2017), Herpes simplex (Aryee et al, 2005), *Staphylococcus aureus* (Huygens et al, 2006), human papillomavirus (Moreau et al, 2013), influenza B (Wong et al, 2014), lymphocystis disease virus (Ciulli et al, 2015), etc. In some cases, the same assay was standardized to simultaneously quantitate and genotype the pathogen (Payungporn 2004, Aryee et al, 2005, Ciulli et al, 2015).

Table S1 presents studies using real-time PCR with SYBR Green to detect, quantitate and genotype parasites from the genus *Leishmania*. Weirather et al (2011) standardized several real-time PCR assays to detect, quantitate and genotype all *Leishmania* species from clinical and experimental samples. These authors analysed 41 *Leishmania*-specific primer sets, with 17 primers targeting the kinetoplast minicircle, 7 primers targeting maxicircle sequences, and 17 primers targeting genes in the nuclear genome. From a combination of SYBR Green and TaqMan-based assays, these authors proposed a workflow with a minimum of 3 serial real-time PCR assays to detect/quantitate and perform parasite identification in the serum samples from patients with visceral leishmaniasis or lesion biopsy specimens of subjects presenting CL.

In 2012, Pita-Pereira *et al* used a set of primers targeting a 120-bp conserved region of kDNA and observed a difference of 1.6 °C between the mean Tm in melting curves derived from the amplification of *Leishmania* from *Viannia* and *Leishmania* subgenera. This genotype assay was validated using 30 clinical specimens from patients with visceral or cutaneous leishmaniasis, and naturally infected sand fly samples from Brazil. For this amplified region in kDNA minicircle, species from the subgenus *Viannia* were analysed (*L. shawi* and *L. braziliensis*), revealing a lower G/C content (43.6%) compared to reference strains from the subgenus *Leishmania, L. infantum* (48.1%) and *L.*

amazonensis (48.7%), which could explain the lower melting temperature observed for DNA amplicons derived from the subgenus *Viannia* (77.34°C ± 0.01). Through the amplification of a 138-bp sequence in the kDNA conserved region, De Morais et al (2016) established two ranges for *Leishmania* genotyping: range 1, with Tm 78 – 79.99 °C, included *L. braziliensis*, *L. panamensis*, *L. lainsoni*, *L. guyanensis* and *L. shawi*; and range 2, with Tm 80 – 82.2 °C, included *L. naiffi*, *L. amazonensis* and *L. mexicana*. The assay was validated with 223 positive blood samples, with 58 samples included in range 1 and 165 samples in range 2, and the genotypes were confirmed using DNA sequencing or RFLP analysis.

De Almeida *et al* (2016) developed and validated a SYBR-Green based assay targeting a 220-275 bp sequence on the ITS1 region of the rRNA gene. In this study, using DNA extracted from reference strains from WHO and CDC isolates, four groups of *Leishmania* parasites were differentiated according to the Tms: group 1 included species from *Viannia* subgenus (*L. braziliensis*, *L. guyanensis*, and *L. panamensis*); group 2A included species from *L. (L.) donovani* spp. complex (*L. donovani* and *L. infantum*); group 2B included *L. tropica* and group 3 included the aggregate species *L. mexicana*, *L. amazonensis*, *L. major*, and *L. aethiopica*. These authors used two distinct real-time PCR systems to evaluate the reproducibility of the assay, with 100% consistency in species identification, although the median Tm values were approximately 1.5 °C higher for each group in one system. This difference highlights the limitation of the comparison of absolute Tm values between different laboratories and real-time PCR instruments. Nevertheless, from the 1,051 patients analysed, this methodology detected parasites in 477 samples, in contrast with the 465 positive results obtained using conventional ITS2-PCR assays as a reference, resulting in improved clinical sensitivity, specificity and species differentiation.

Employing the widely used set of primers described by Mary *et al* (2004) and a new primer pair (MLF and MLR), both standardized for SYBR green-based assays, Ceccarelli et al (2014) described a methodology to perform simultaneous quantification and species identification in the same reaction, targeting the conserved region of kDNA minicircle. Linearity from 10^4 to 10^{-3} *L. infantum* equivalents was reported for parasite load quantification in canine

peripheral blood and conjunctival swabs samples. Consistent with the previously reported results, this assay could discriminate between *L. (Leishmania)* and *L. (Viannia)* subgenera. In addition, the number and genetic variability of kDNA minicircles in *L. infantum* WHO international reference strain was investigated. The heterogeneity of minicircles in the reference strain and canine clinical samples was observed, highlighting the limitations of standard curve construction for absolute quantification using real-time PCR.

High Resolution Melting (HRM) is a method for genotyping, mutation scanning and sequence matching, in which no PCR product separation or sample processing is required. After DNA target amplification using PCR, melting curves are generated, monitoring the fluorescence of a saturating dye that does not inhibit the PCR (Reed et al, 2007). HRM assays are enabled by the new generation of saturating dyes, such as Eva Green or SYTO9, together with instruments dedicated to HRM or adapted from real-time PCR equipment. These instruments, such as ViiA7 and QuantStudio (Applied Biosystems) or Rotor-Gene 6000 (Corbett), are capable of fluorescence monitoring at a 0.017 °C/s rate, producing 10 reading points/°C. In leishmaniasis, this methodology was explored in two recent studies: Hernandez et al (2014) and Zampieri et al, 2016. The former standardized an HRM assay targeting *Leishmania* HSP70 and ITS1 genes. According to a proposed algorithm, six *Leishmania* species from the New World (*L. mexicana*, *L. infantum*, *L. amazonensis*, *L. panamensis*, *L. guyanensis* and *L. braziliensis*) were discriminated from the reference strains and 35 parasite isolates, from human, insect vectors and mammals, with a limit of detection corresponding to 10 parasite equivalents/mL. In the latter study, the authors developed an assay targeting two different regions of *Leishmania* HSP70 coding gene: a 144-bp region amplified in all *Leishmania* species and a 104-bp region amplified only for species from *L. (Viannia)* subgenus. Based on the HRM curves analysis, this assay discriminated eight *Leishmania* species found in the Americas (*L. infantum*, *L. amazonensis*, *L. mexicana*, *L. lainsoni*, *L. braziliensis*, *L. guyanensis*, *L. naiffi* and *L. shawi*) and three species found in Eurasia and Africa (*L. tropica*, *L. donovani* and *L. major*). The assay was validated with sixteen tissue samples from infected Golden Hamsters, parasite strains isolated from human samples, human fresh and paraffin-embedded tissues and tissues from infected BALB/c mice and naturally infected

phlebotomies. HRM results were compared with previous genotyping by sequencing of SSU rDNA or PCR targeting g6pd, presenting high correlation between methodologies.

Tsukayama et al (2013) developed a FRET-based real-time PCR assay with LightCycler™ probes (HybProbes) targeting *Leishmania* MPI and 6PGD genes, to diagnose and characterize New World tegumentary leishmaniasis (TL) from tissue samples. According to this methodology, *L. braziliensis, L. panamensis L. peruviana, L. guyanensis, and L. lainsoni* were differentiated after combining MPI and 6PGD primers/probes sets, through specific melting curves generated using LightCycler probes. The methodology was validated using 192 specimens belonging to the same number of individuals with leishmaniasis-like lesions (178 biopsy and 14 lesion scraping samples), attended in two reference centres for leishmaniasis diagnosis and treatment in Lima, Peru. When a conventional PCR targeting kDNA was used as the gold standard, 92% sensitivity and 77% specificity were achieved, and positive and negative predictive values were 97% and 59%, respectively. Remarkably, compared to MLST, this methodology was three times faster and five times less expensive for *Leishmania* species identification. Most recently, Nath-Chowdhury et al (2016) developed a similar assay with LightCycler HybProbes, targeting the cathepsin L-like cysteine protease B (cpb) gene from *Leishmania*. In this study, these authors simultaneously identified three species of Old World cutaneous leishmaniasis, *L. major*, *L. tropica*, and *L. aethiopica* based on the melting curves using a single primers/probes set. The methodology was validated using 28 DNA samples from *L. major*, *L. tropica*, and *L. aethiopica* promastigotes from reference strains and seven lesion biopsy samples of patients with cutaneous leishmaniasis. The primers for the *cpb* gene amplified *L. infantum* and *L. donovani* with non-specific low-melting peak temperatures and did not amplify New World *Leishmania* species and non-leishmanial parasites.

Taken together, real-time PCR application for *Leishmania* species identification represents an advance to classic genotyping methodologies, with a significant reducing in costs per assay, high sensitivity and less time-consuming. However, a simple and feasible assay to simultaneously estimate parasite load and genotype all species of interest according to the geographical

region and clinical manifestation is still necessary, particularly for CL in the Americas. Despite many protocols that enable *Leishmania* species discrimination at some level, multi-centric studies are still needed as a validation step.

Current challenges for the molecular diagnosis and parasite load quantification in CL

The diagnosis of leishmaniasis is challenging because of the wide spectrum of clinical manifestations (Reithinger & Dujardin, 2007). For CL, skin lesions vary in severity, clinical appearance, duration, and in some cases, can be indistinguishable from lesions caused by bacteria and fungi, such as cutaneous botryomycosis, lepromatous leprosy, cutaneous lobomycosis, or cancer. Moreover, in some endemic countries of Latin America, co-infection with other trypanosomatids, such as *Trypanosoma cruzi*, can lead to false-positive results depending on specificity of diagnostic method.

Moreover, in some endemic countries of Latin America, mixed infection with other trypanosomatids can lead to false-positive results depending on the specificity of diagnostic method. Although studies regarding this important matter are rare, mixed infections caused by *Leishmania* sp. and *T. cruzi* have been reported in patients with TL, ranging between 12% and 70% (Frank et al., 2003; Chiaramonte et al., 1999, Gil et al., 2011; Vega Benedetti et al., 2013). In a case-control study in endemic areas for TL and Chagas disease, at the region of Salta (Northeast of Argentina), Hoyos et al (2016) observed a prevalence of 0.17% for TL, a 9.73% infection seroprevalence for *T. cruzi*, and 16.67% mixed infection proportion within the *Leishmania* patients group. In sylvatic reservoirs, Araujo *et al.* (2013) provided the first description of a triple mixed infection, by *T. cruzi*, *Trypanosoma rangeli* and *L. (L.) infantum (chagasi)*, in a bone marrow sample from an anteater *Tamandua tetradactyla*. The mammalian host was captured in a house backyard from a sympatric area for these trypanosomatid species, endemic for Chagas disease and VL in the Amazon basin, Brazil. Additionally, mixed infections can lead to therapeutic implications since the treatment of leishmaniasis with antimonial drugs can present cardiac toxicity, which is highly restrictive to Chagas disease patients with chronic Chagas cardiomyopathy. These studies highlight the

epidemiological importance of mixed infections in endemic areas and the need for the development of molecular assays with high sensitivity and specificity to the Leishmaniasis diagnosis.

Since the advent of PCR in the 1980's, a high number of molecular assays have reported the detection and identification of *Leishmania* parasites DNA with high sensitivity and specificity (Schönian et al 2003; Garcia et al. 2004; Bensoussan et al. 2006; Nasereddin et al. 2008; Graça et al, 2012. More recently, quantitative PCR assays have been developed to estimate parasite load from blood, tissue samples and skin or mucosal lesions in patients with leishmaniasis. Nevertheless, as shown in Table S1, despite the high number of assays, there is still no consensus regarding the sample harvesting methods, DNA extraction, molecular targets, and the use of endogenous and exogenous quality controls and data normalization. Notwithstanding, the number of studies applying real-time PCR for diagnostic and parasite quantification in CL patients and the heterogeneity of methodological approach hinder the comparison of these studies, and thus so far it is not possible to indicate the best conduct.

Detection and quantification assays for of *Leishmania* using PCR in CL are often developed and employed at hospitals or research centres. For the most reported in-house assays, international guidelines for analytical and clinical validation were not followed, and the performance of reactions cannot be properly evaluated. The Centres for Medicare & Medicaid Services (CMS) regulate all laboratory testing performed on humans in the United States of America through Clinical Laboratory Improvement Amendments (CLIA) of 1988. CLIA regulations ensure accuracy, reliability, and appropriateness of clinical test results, setting minimum standards applied in validating the performance of clinical tests, but with different recommendations between the implementation of FDA (Food and Drug Administration, USA)-approved tests and laboratory-developed tests. Accordingly, performance characteristics must be verified for laboratory-developed tests, including accuracy, precision, reportable range, reference interval, analytical sensitivity and analytical specificity, following well-detailed procedures to perform each test (Burd, 2010).

Most of the PCR assays for leishmaniasis have been developed to detect/quantify a single or few *Leishmania* species and are not appropriate for regions with several sympatric species (Akhoundi et al, 2017). Even in areas

where circulating *Leishmania* species are known, intra-species genetic variation, as the accumulation of polymorphisms and variations in gene copy number, could interfere with the performance of these assays, particularly in quantitative real-time PCR. In the Americas, the prevalence of *Leishmania* species causing CL is well known in most endemic areas. Nevertheless, a consensus methodology comprising a DNA extraction protocol with exogenous quality control, coupled to a multiplex quantitative real-time PCR assay targeting a highly conserved region of a *Leishmania* target gene and a human gene is still needed. The human gene will serve as an internal reference to normalize the parasite load based on the amount of human DNA. The analytical and clinical performance of this consensus methodology must be properly evaluated according to recommendations of international guidelines. Moreover, multi-centric studies are necessary to establish standardized operative procedures that could be applied to leishmaniasis (Schijman et al., 2016). Considering the absolute quantification using real-time PCR, the establishment of well-defined standard curves is necessary to increase the precision of these assays. Figure 1 summarizes the tentative steps towards the standardization and validation of a real-time PCR-based methodology for the molecular diagnosis of CL. Considering the variability in species and strains of *Leishmania species* circulating in CL endemic areas, a standardization initiative is essential to guide researchers and clinical laboratories into a robust and reproducible methodology, enabling further evaluation of parasite load as a prognosis biomarker and the monitoring of etiological treatment, particularly in multi-centric observational studies and clinical trials.

TDR/WHO technical report on Research Priorities for Chagas disease, HAT, and leishmaniasis emphasized the need for simplifying and standardizing application of PCR in the field and highlighted the need for validation and reliability studies (WHO, 2012). In response to this concern, the Pan-American Health Organization is promoting discussions to identify possibilities for method development, standardization, quality control and laboratory validation of quantitative PCR across laboratories. Furthermore, an international initiative has been launched to develop standardized operative procedures for real-time PCR-based molecular diagnosis of CL. In December 2016, an international workshop was held in Medellín, Colombia, with the participation of ten groups

with previous experience in PCR for CL, from seven countries of Latin America (Argentina, Brazil, Colombia, Costa Rica, Mexico, Panama and Peru). This workshop aimed to compare the performance of molecular assays for the detection and quantification of *Leishmania* species of interest, to establish a consensus in a multiplex real-time PCR protocol with one target in *Leishmania* and one target in human DNA, to the absolute quantification of parasite load with normalization based on the human DNA content from skin lesion samples. From the workshop, a methodology to preserve the skin lesion samples during the harvesting and transportation coupled to a silica-column based protocol for DNA extraction containing an exogenous internal positive control was standardized. In addition, three real-time PCR assays targeting SSUrDNA, kDNA, and HSP70 from *Leishmania*, in multiplex with human RNAse P gene were compared. The subsequent analytical and clinical validation of these assays is needed, according to the specifications of international guidelines. To this end, DNA extracted from skin lesion samples obtained from CL patients attending health facilities in the participating countries will be employed.

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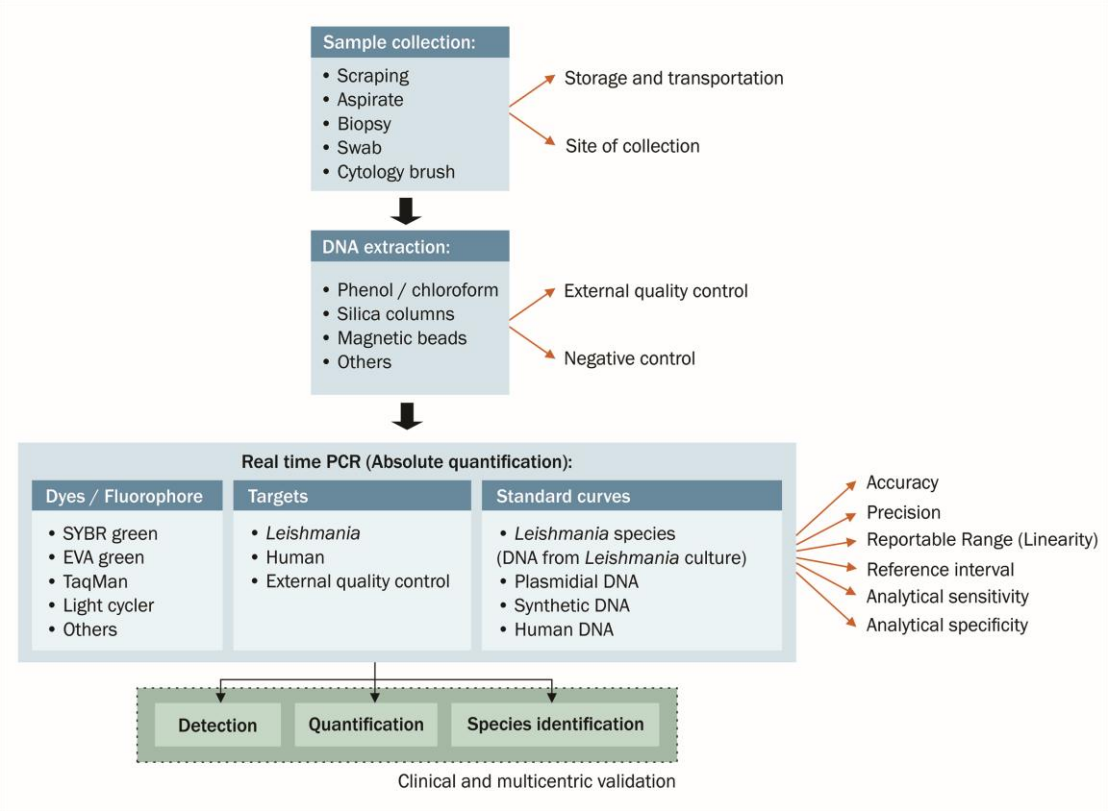

Figure 1. Workflow for standardization and validation of a real-time PCR-based methodology for the molecular diagnosis of CL. The workflow indicates the alternatives in each phase of the molecular method, inside the blue boxes. On the right side, the orange arrows indicate the steps to be standardized during sample collection and DNA extraction, and the validation steps for the real-time PCR assay. The dashed green box indicates the main applications of this methodology for CL, highlighting the necessity of a clinical and multi-centric validation for this stage.

Table 1. Summary of the characteristics of real-time PCR assays targeting kDNA, SSUrRNA and HSP70 for *Leishmania* detection.

Targets	Assay type		Assay dye/fluorophore			Internal reference		Leishmania identification/ discrimination	
	Quantitative	Qualitative	TaqMan	SYBR Green	Others	Yes	No	Yes	No
kDNA	10	2	5	9	0	5	7	5	7
SSUrRNA	8	3	3	5	3	6	5	6	5
HSP70	1	2	1	1	2	1	2	3	0
Others	10	4	10	8	3	13	1	12	2
Total	29	11	19	23	8	25	15	26	14

Note: for more details, see Table S1