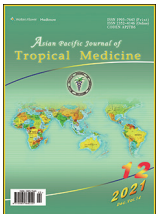

Review Article

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Methods and parameters of melting curve analysis for identification of *Leishmania* species: A scoping review

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ABSTRACT

Leishmaniasis is a set of diseases with a worldwide distribution that affects mainly economically underprivileged populations in developing countries. It has a major impact on public health, with a global cost of billions of dollars per year. The treatment and control of leishmaniasis vary according to the *Leishmania* species involved, which require reliable methods for species identification. Since most of the currently used methods have limitations, there is a need for assays that allow rapid, precise identification of the offending species. Real-time polymerase chain reactions in conjunction with dissociation curve analysis have been used to detect differences in the DNA composition of selected genes of *Leishmania* spp. Kinetoplast DNA is the main molecular target used because of its high copy number per parasite, but other targets have also been studied. As part of an effort to establish melting temperature standards for each target gene, we have reviewed the pertinent literature available in public databases, including PubMed, Web of Science, SciELO and LILACS, using the keywords "*Leishmania*", "leishmaniasis", "real-time PCR", "melting temperature", and "melting curve", alone or in combination. After applying eligibility criteria, 27 articles were selected for analysis. A considerable variation in the methodologies analyzed was found regarding molecular targets, standardization of the methods, reproducibility and specificity. Because of this, statistical analysis was not performed. In most cases, the methods were able to differentiate the parasite at the subgenus level or few species regardless of the target chosen.

KEYWORDS: Leishmaniasis; Melting temperature; Real-time PCR

1. Introduction

Leishmaniasis is a vector-borne disease caused by parasites of the genus *Leishmania* that are transmitted by phlebotomine insects to a variety of mammals, mainly humans and domestic animals such as dogs. *Leishmania* parasites are classified into two subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*), with the clinical symptoms of the disease varying according to the offending species. The main forms of the disease are visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucosal leishmaniosis (ML). Globally, leishmaniasis is associated mainly with poverty and affects more than 12 million people, with 0.9 to 1.6 million new cases per year, estimated yearly deaths between 20 000 and 30 000, approximately 350 million people are at risk of infection[1]. In the Americas, CL and ML are endemic in 18 countries, 17 of which reported 892 846 new cases of CL from 2001 to 2016; VL occurs in 76 countries (12 of which are located in the Americas) and, in the same 15-year period indicated above, 55 530 cases of this form of leishmaniasis were reported (mean of 3 457 cases/year)[2].

The wide variety of species involved in leishmaniasis, together with the large spectrum of clinical manifestation, makes the diagnosis of this disease complex, particularly since the various clinical symptoms require different therapeutic approaches.

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Visceral leishmaniasis is the systemic and the most severe form of the disease, leading to death if untreated. The most common features include fever, splenomegaly, hepatomegaly, pancytopenia, anemia, and weight loss, but VL may also be asymptomatic. In addition, the clinical symptoms and manifestations may vary among geographic regions, as may the treatment used, primarily because of different patterns of parasite resistance to medicines[3]. The drugs currently recommended by the World Health Organization (WHO) includes amphotericin B, miltefosine, paromomycin, and pentavalent antimonials (or combination of these), depending on the case or region[4].

The initial symptom of CL is a papule or nodule at the site of the infection that subsequently progresses to ulcerated nodules. CL has some rare variations, such as diffuse CL, disseminated CL and CL relapse, that are associated with the immune responses[5]. The type and severity of lesions and their spontaneous healing also depend on the immune response, in addition to the *Leishmania* species responsible for the infection[4–6]. The choice of treatment will depend on the species causing Old World or New World CL infection, as well as on the availability of medicines, thermotherapy and cryotherapy[4].

ML, which is characterized by destructive lesions of the nasal septum, lips, and palate, occurs mainly in immunocompromised subjects and requires rapid treatment to avoid permanent disfigurement[5]. ML is generally treated with pentavalent antimonials, amphotericin B or miltefosine, with the latter being used only in certain countries[4].

2. Diagnosis

The standard visualization of amastigote forms of *Leishmania* in diverse biological samples shows high specificity, but is unable to distinguish the species[4]. Serological techniques, although useful for screening in endemic areas, do not distinguish the species or the status of infection. Detection of the parasite DNA by polymerase chain reaction (PCR) has high sensitivity and specificity, and certain variations of this method, such as restriction fragment length polymorphism (RFLP-PCR), allow identification of the species[7,8]. The gold standard for typing *Leishmania* species and strains is multilocus enzyme electrophoresis (MLEE), introduced in 1990 by Rioux *et al.*, for classification of the genus *Leishmania*[9]. This method identifies the parasites by their enzymatic profile (known as zymodemes) based on their electrophoretic mobility, and several enzyme panels can be analyzed simultaneously. The use of MLEE is limited because of the large amount of proteins required that can only be obtained by culturing *Leishmania*[10].

Real-time PCR (qPCR) with molecular probes or an intercalating dye can be used to detect the parasite and provide quantitative results. SYBR Green® technology, which uses an intercalating dye, allows melting curve analysis (MCA) of the dissociation profile of double-stranded DNA[11]. High-resolution melting analysis (HRMA) fulfills the same function but provides better performance with regard instrumentation and the fluorescence of DNA-binding dyes[12,13]. Both qPCR and HRMA are used for various applications in microbiological and parasitological analysis, as well as to discriminate *Leishmania* subgenera and/or species[14–20].

The choice of the molecular target is an important issue for accurate parasite identification by PCR because of the wide variety of targets available. The kinetoplast DNA (kDNA) is one of the most studied targets among species of the class Kinetoplastida because of its peculiar organization in maxicircles and minicircles, with the latter being highly conserved. Since minicircles are very abundant, specific and repetitive, they have been used as a molecular target to detect and identify *Leishmania* species by different PCR strategies.

Targets other than kDNA have also been used. For example, the ribosomal internal transcribed spacer 1 (*ITS1*) is a region with a high number of copies of the *rRNA* gene and shows interspecific variations and single nucleotide polymorphisms (SNPs)[16,21]. *HSP70*, a heat-shock protein with an important role in cellular and protein interactions, has been used for the molecular identification of *Leishmania* and for phylogenetic studies because of its conserved nature[22,23]. The tryparedoxin peroxidase gene has antioxidant activity that contributes to parasite viability. This gene is located on chromosome 15 and has three copies in the form of a tandem repeat that allow detection of even low levels of expression[24]. Cathepsin L-like cysteine proteinase B is encoded by the gene for *Cpb*, a major antigen of *Leishmania* parasites. This gene, which is highly conserved, occurs in multiple copies among species, is polymorphic and is a sensitive target for parasite detection[25,26]. Concomitant sequencing of mannose phosphate isomerase (*MPI*) and 6-phosphogluconate dehydrogenase (*6PGD*) genes was able to differentiate seven species of New World *Leishmania* because the genetic polymorphisms involved were species-specific[27,28]. Other genes that have been used include that of the single-copy, housekeeping *Leishmania* RNA-binding protein (*DRBD3*), which is considered ideal to this purpose[29].

Together, these studies indicate that a wide variety of targets has been used for species identification in human and animal samples, with marked inter-specific differences being observed in melting temperature (Tm) analysis, even when using the same target. The large variety of biological samples that can be used for DNA extraction may also influence the assay sensitivity.

Based on the foregoing studies, the objectives of this investigation were to: 1) review the use of Tm for *Leishmania* identification in humans, dogs, mice and/or cultured parasites; 2) analyze the Tm of each *Leishmania* species, according to the gene/target chosen; 3) analyze the criteria used to assess method quality; and 4) analyze the procedures for method standardization.

3. Literature searching on melting curve analysis and findings

Literature searches of four public databases (PubMed, Web of Science, Scielo and LILACS) were done in May, 2020. The keywords used for search were "*Leishmania*", "leishmaniasis",

"real-time PCR", "melting temperature" and "melting curve", alone or in combination, using the Boolean operators "AND" and/or "OR". Search was performed in duplicate and papers were selected after reading the title and/or abstract and assessing whether they met the inclusion criteria. In addition, the reference lists of studies included in the review were carefully scanned for possible articles not retrieved from the databases.

The eligibility criteria used were: 1) articles written in English, Spanish or Portuguese idiom; 2) publication within the period from January, 2000 until April, 2020; 3) studies that involved humans, dogs, mice and/or cultured parasites; 4) studies done in any country; and 5) studies that used real-time PCR to describe Tm or HRMA for different *Leishmania* subgenera or species. Studies that exclusively analyzed phlebotomine insects and other mammals, review articles, and articles not containing a precise description of the method and the Tm found were excluded.

The data obtained were classified into different variables and organized in tables to support the interpretation of data analysis (Tables 1-5). In addition, the Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR)[30] was applied to identify and report the data.

This study involved a descriptive analysis of the Tm for each *Leishmania* species, in addition to each target/gene analyzed. The wide variation among techniques meant that the application of meta-analyses was impracticable.

The literature search identified 1 312 references, of which 1 285 were excluded because they did not meet the inclusion criteria or were duplicates. A total of 27 references that used of MCA and or HRMA for identification of the *Leishmania* species or subgenus were selected (Figure 1).

Table 1. Distribution of species in the subgenera *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*).

Visceral or cutaneous leishmaniasis *Leishmania* (*Viannia*)	Cutaneous or mucosal leishmaniasis *Leishmania* (*Leishmania*)	
	New World	Old World
Leishmania braziliensis	*Leishmania infantum chagasi*	*Leishmania infantum infantum*
Leishmania guyanensis	*Leishmania mexicana*	*Leishmania major*
Leishmania panamensis	*Leishmania pifanoi*	*Leishmania tropica*
Leishmania shawi	*Leishmania venezuelensis*	*Leishmania killicki*
Leishmania naiffi	*Leishmania garnhami*	*Leishmania gerbelli*
Leishmania lainsoni	*Leishmania amazonensis*	*Leishmania aetiopica*
Leishmania lindenbergi		
Leishmania peruviana		
Leishmania colombensis		

The two methods most widely used in the studies were MCA and HRMA. Of those that used MCA, five employed integrated fluorescent probes in conjunction with fluorescence-resonance energy transfer (FRET; referred to hereafter as FRET/MCA).

These methods were applied for different subjects: cultured parasites, humans, mice, dogs, cats and insects; however, we only included the analysis samples from parasites, humans, mice and dogs. Samples were also diverse: blood, buffy coat, biopsy, cutaneous lesions, dermal scrapings, bone marrow aspirate, urine and conjunctival swab.

To assess the quality of qPCR validation, we identified researches that performed tests of sensitivity (18/27, 66.6%), specificity

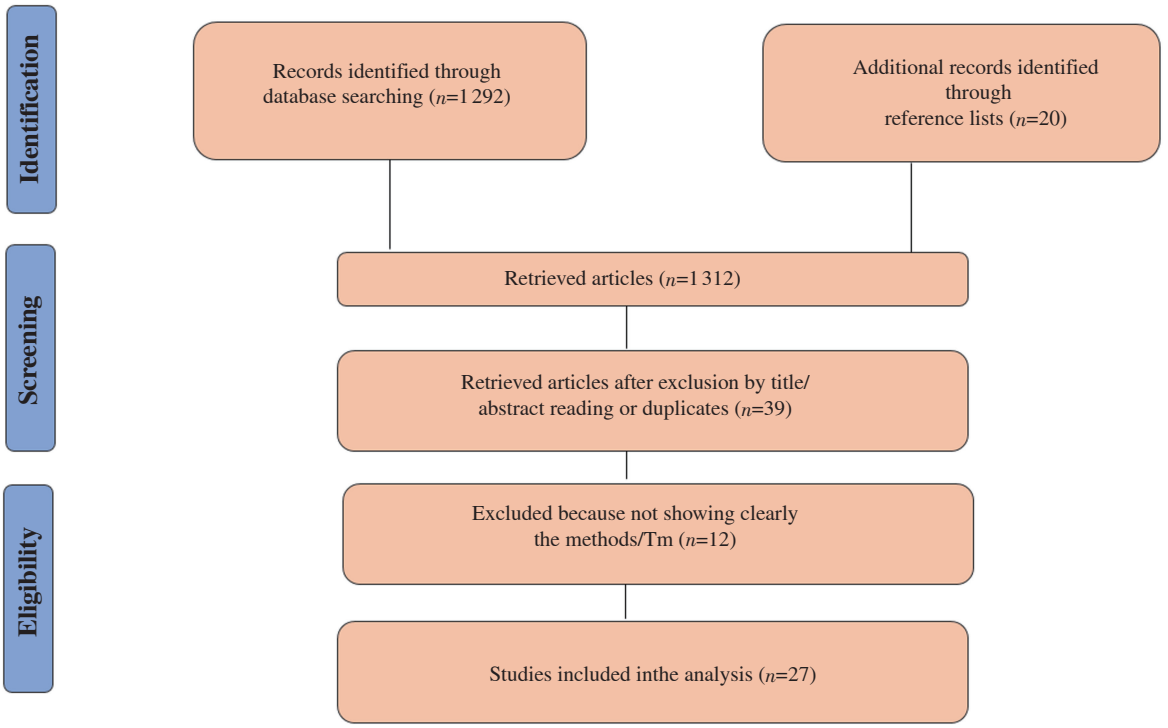

Figure 1. Flowchart of the inclusion criteria used in this study.

(17/27, 62.9%) and reproducibility (12/27, 44.4%). In addition, it was analyzed which studies estimated DNA concentration (14/27, 51.8%), which used replicates (17/27, 62.7%), which ran independent reactions (17/27, 62.7%), which used serial dilutions (21/27, 77.7%), which used intern control (6/27, 22.2%) and which verified cross-reactions with other microorganisms (14/27, 51.8%) (Table 2). The confirmation of *Leishmania* species found through sequencing or cloning was performed in 59.2% (16/27) and 25.9% (7/27), respectively. Incomplete analysis of all method validations represent the study limitation which hinders a comparative analysis among them.

A detailed description of primer sequences and Tm for each *Leishmania* specie according to molecular target are discriminated in Tables 3 and 4. The main characteristics of selected studies as first author, year of publication, country, population, molecular target, methodology, main objective and outcome are available in Table 5.

Few papers reported study limitations or risk of bias for the sample included in its analysis. The main reported issues were the sample size, absence of multicentric studies, absence of negative samples resulting in imprecise negative predictive value and the impossibility to apply all tests to all study participants.

Identification of the species causing the disease is a crucial step for better management and treatment, as well as for possible control measures. In addition, adequate methods for species identification are essential for eco-epidemiological studies since they allow determination of the species circulating among phlebotomies in domestic and wild reservoirs[4].

Reference strains or cultured isolates, previously identified by MLEE and/or other methods such as PCR-RFLP or single target sequencing, were listed in 59.3% of the reports. Although most of the studies mentioned the use of species in culture, in several cases the method used for species identification was unclear. In addition to the use of cultured promastigotes, specimens obtained from a variety of sources (peripheral blood, bone marrow, tissue biopsy, skin lesion fluid, skin lesion scrap, urine) from humans and/or animals (dogs, cats, mice) were also tested. Four studies used filter paper to improve sample collection and preservation[15,31–33]. However, this procedure was less invasive and proved useful for field studies, two reports found it to be less sensitive when compared with the use of directly collected specimens[15,32]. In 51.8% of the reports, the concentration of DNA extracted from different samples were analyzed (Table 2). Several authors analyzed the interference of template DNA concentration[18,33–39] and host DNA[34,36,37,39] on the variation in Tm, but only a few observed any shift in the melting curve in relation to the template[18,36] and host DNA concentration[18,39]. Although these variables do not appear to affect all *Leishmania* spp., they should nevertheless be carefully evaluated since they could lead to species misidentification, especially when using HRMA that allows the use of a smaller Tm difference to distinguish among species.

Although quantitative PCR has been widely used as a diagnostic tool, the nomenclature and concepts used have not always been applied appropriately, including in some of the articles examined here. According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines[40], the correct abbreviation for quantitative PCR is qPCR, while RT-PCR refers to quantitative reverse transcription PCR. Two reports used the abbreviation RT-PCR to refer to real-time PCR[24,41].

Analytical sensitivity is the "minimum number of copies that can be measured accurately with an assay, generally known as the limit of detection (LOD)", while clinical sensitivity is "the percentage of individuals with a given disorder whom the assay identifies as positive for that condition". Analytical specificity is "the detection of the appropriate target sequence rather than other, nonspecific targets also present in a sample" and diagnostic specificity is "the percentage of individuals without a given condition whom the assay identifies as negative for the condition". Repeatability is the intra-assay variation, or the precision of the same samples evaluated in one assay, while reproducibility is the inter-assay variance, or the variation in results among different runs or laboratories[40]. These definitions are very important for guaranteeing the homogeneity of analyses. However, given the variation in the methods and analyses reported in the different studies, it was not possible to compare several of these aspects.

The test sensitivity was calculated in 66.6% of the articles (Table 2), and in 77.7% of these was tested by serial dilutions, with a minimum of 0.001 parasite/reaction being detected by Ceccarelli *et al.* using kDNA[42]. By using plasmids in a duplex method that targeted *MPI* and *6PGD* genes, Tsukayama *et al.* were able to increase the assay sensitivity to detect as little as 10-100 copies of parasite DNA[27].

Test specificity was calculated in 62.9% of the articles, although only 51.8% of the studies assessed cross-reactivity. More than 23 species were screened, with *Trypanosoma (T.) cruzi* and *T. brucei* being the most used trypanosomatids. In one study, *T. cruzi* DNA was detected by kDNA amplification (Tm: 81.5 ℃), but this Tm did not overlap with *Leishmania* species. This interesting finding could be used to differentiate leishmaniasis from Chagas disease in areas where both diseases are endemic since the current serological tests used to distinguish between them may show cross-reactivity[43,44]. The use of an internal control to detect inhibitors in DNA samples was reported in only six studies (Table 2). These controls were used to confirm DNA quality and ensure that false negative results were not attributable to the low quality of the samples, and. When these controls are not used, negative results are unreliable and may affect the clinical sensitivity.

Although 17 studies used replicates to calculate the standard deviation for each Tm, most of them did not specify how many replicates were run. Fifteen reports calculated the reproducibility by performing more than one run for the same samples or control and the remaining did not calculate this parameter, did not specify how they calculated it or calculated it using a positive control in the same run (Table 2).

A range of Tm values was reported for the same species of *Leishmania* and even for the same targeted sequence. Table 4 lists all the *Leishmania* species, targets and Tm, as well as the PCR

Table 2. Assay parameters in studies of melting point analysis.

Reference	Sequencing	Cloning	DNA conc.	Filter paper	Sensitivity	Specificity	Reproducibility	Ref. strain	Replicates	Independent	Serial dilutions	Internal control	Cross reaction
Diotallevi *et al*, 2020[15]	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes
Almeida *et al*, 2017[16]	Yes	No	Yes	No	No	No	No	Yes	No	No	No	Yes	Yes
Hernández *et al*, 2014[17]	No	No	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	No
Zampieri *et al*, 2016[18]	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes
Khosravi *et al*, 2012[24]	No	No	No	No	Yes	Yes	Yes	No	No	Yes	No	No	No
Nath-Chowdhury, 2016[25]	Yes	No	No	No	Yes	Yes	No	Yes	No	No	Yes	No	Yes
Tsukayama *et al*, 2013[27]	Yes	Yes	No	No	Yes	Yes	No	Yes	No	No	Yes	Yes	Yes
De Monbrison, Mihoubi, Picot; 2007[31]	Yes	No	No	Yes	Yes	No	No	No	No	NS	Yes	No	No
Eroglu, Uzun, Koltas; 2014[32]	Yes	No	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	No	No
Nasereddin, Jaffe; 2010[33]	Yes	No	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes
Khademvatan *et al*, 2011[35]	Yes	No	No	No	No	No	No	No	No	Yes	Yes	No	No
Ceccarelli *et al*, 2014[36]	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes
Ceccarelli *et all*, 2017[37]	Yes	No	No	No	No	Yes	No	Yes	Yes	Yes	No	No	Yes
Kuang *et al*, 2017[38]	No	No	No	No	No	Yes	Yes	No	Yes	Yes	Yes	No	No
Müller *et al*, 2018[39]	No	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes
Abda *et al* 2011[41]	No	No	No	No	No	No	Yes	Yes	No	NS	No	No	No
Mohammadiha *et al*, 2013[62]	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Pessoa-e-Silva *et al*, 2016[48]	No	No	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	No
Schulz *et al*, 2003[52]	No	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Tupperwar *et al*, 2008[49]	Yes	No	Yes	No	Yes	No	Yes	No	Yes	Yes	Yes	No	No
Pita-Pereira *et al*, 2012[46]	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	No	No	Yes
Toz *et al*, 2013[63]	Yes	No	Yes	No	Yes	No	Yes	Yes	No	Yes	No	No	No
De Morais *et al*, 2016[47]	Yes	No	Yes	No	No	No	No	Yes	Yes	Yes	No	No	No
Nicolas, Milon, Prina; 2002[45]	No	No	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes
Cavalcanti *et al*, 2013[51]	Yes	No	Yes	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes
Marin *et al*, 2017[57]	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes
Quispe-Tintaya *et al*, 2005[53]	No	Yes	No	No	No	No	Yes	Yes	Yes	No	Yes	No	No

NS: not specified; Conc.: concentration; Ref.: Reference.

Table 3. Primer and nucleotide sequences of the target genes analyzed.

Target	Primers	Sequence	References
kDNA	*JW13* *JW14*	5'-ACTGGGGGTTGGTGTAAAATAGG-3' 5'-TTTCGCAGAACGCCCCTACCC-3'	[31,35,41,45]
	N.S. N.S.	5'-GGC CCA CTA TAT TAC ACC AAC CCC-3' 5'-GGG GTA GGG GCG TTC TGC GAA-3'	[46]
	kDNAf *kDNAr1* *kDNAr2*	5'-ATGCCTCTGGGTAGGGGCGTTC-3' 5'-GGGAGCGCGGCCCACTATATT-3' 5'-GGGTGCGCGGCCCACTATATT-3'	[51]
	kDNAf *kDNAr1*	5'-ATGCCTCTGGGTAGGGGCGTTC-3' 5'-GGGAGCGCGGCCCACTATATT-3'	[47]
	MLF *MLR*	5'- AAA ATG AGT GCA GAA ACC C-3' 5'-CGGCCCTATTTTACACCAACC-3'	[15,36,37]
	RV1 *RV2*	5'-CTTTTCTGGTCCTCCGG- GTAGG-3' 5'-CCACCCGGCCCTATTTTACACCAA- 3'	[62]
	LInf.1–23F *Linf.1–154R*	5'-TCCCAAACTTTTCTGGTCCT-3' 5'-TTACACCAACCCCCAGTTTC-3'	[48]
Hsp70	*HSP70F* *HSP70R*	5' AGG TGA AGG CGA CGA ACG-3' 5' CGC TTG TCC ATC TTT GCG TC-3'	[17]
	hsp70C *hsp70F2* *hsp70F1* *hsp70R1*	5'-GGA CGAGATCGAGCGCATGGT TCCTTCGACGCCTCCTGGTTG-3' 5'–GGAGAACTACGCGTACTCGATGAAG–3' 5'–AGCGCATGGTGAACGATGCGTC–3' (5'–CTTCA TCGAGTACGCGTAGTTCTCC–3'	[18]
ITS1	*LITSR* *LITSR* *L5.8S* *ITS1f* *ITS1r*	5'- CTGGAT CAT TTT CCG ATG-3' 5' -TGA TAC CAC TTA TCG CAC TT-3' 5'-GTAACAAGGTAGCTGT-3' 5'-TTCTACTGTATGTTGT-3'	[17,32] [51]
	LITSR *ITS1R–TR1* Probe1 Probe 2	5'-CTGGAT CAT TTT CCG ATG-3' 5'- GAAGCCAAGTCATCCATCGC-3' 5'-CCGTTTATACAAAAAATATACGGCGTTTCGGTTT-3' 5'-GCGGGGTGGGTGCGTGTGTG-3'	[54]
	LSGITS1–F1 *LSGITS1–R1*	5'-CATTTTC CGATGATTACAC-3' 5'-CGTTATGTGAGCCGTTATC-3'	[16]
SSU rRNA	*SSUrDNAf* *SSUrDNAr*	5'-CGACAAGCGCTCTCCC-3' 5'-CCCAGTACGTTCTCCC-3'	[51]
Lack gene	*LACK 1F* *LACK 1R* *LACK 2F* *LACK 2R*	5'-GCGAAGGAAACCAGTGTTTGT-3' 5'-GCTAGCAACAACAGACATCAACTCT-3' 5'-3'CGCAAGTTCCTGAAGCACACC-3' 5'-3'CTCACCCGCCACGTTCCACA-3'	[38]
Amino acid permease 3 (*aap3*)	*AAP3–Amp1–F* *AAP3–Amp1–R* *AAP3–Amp2–F* *AAP3–Amp2–R* *AAP3–Amp3–F* *AAP3–Amp3–R*	5'-ATCCGCTACGTCTCCGCCATCGG-3' 5'-CGTGGTGAAGTACTTCATGTCGC-3' 5'-GCCGTCGATAAACACCCGAGC-3' 5'-AAGCGGAAGATGATGTTGCGCCC-3' 5'-GGCGGTCGCCTACATCAGCG-3' 5'-CGGGCACCATGAACACGAGCCATA-3'	[39]
Gp63	*TV255* *TV256*	5'-AGTA- CGGCTGCGACACCTTGGAG-3' 5'-GTTCCGG- CCCCACGGCATCACC-3'	[49]
7SL RNA gene	*CJ7SLF* *QRT7SLR*	5'-ACG TGG ACC AGC GAG GGT-3' 5'-CGG TTC CCT CGC TTC AAC-3'	[33]
Mannose phosphate isomerase (*MPI*)	*MPI.ext.F* *MPI.ext.R*	5'-CCC TTT GGT TGT CGG T-3' 5'-TCA TAC GCA TAG GAG CA-3'	[27]
6-Phosphogluconate dehydrogenase (*6PGD*)	*6PGD.909.F* *6PGD.1537.R*	5'-CAA GGC GTT CCC TAC ATT C-3' 5'-TTG CGG TCG GGA CAA CTGG-3'	[27]
Tryparedoxin peroxidase gene	*TRYP–F* *TRYP–R*	5' GGGAATTCCATATGTCCTGCGGACGCCAAG-3' 5'CGCGGATCCTACTTGTTGTGGTCGACCTTCATGC-3'	[24]
cpb	*cpb F* *cpb R* *cpb* sensor 2 *cpb* anchor 2	5'-CGGCARCATCGAGTCGC-3' 5'-GGTCCCGTTCATGTTTCG-3' 5'-TCGAACGCCTGCAGCATC-3' 5'-GCCCGCCGCCGCAA-3'	[25]
	7forUniv *Arev8* *PanchoCPB* *Dos12.1*	5'-TGaGGTTCCGTACTGGGTG-3' 5'-GGACCAAAGCAATGAGGG-3' 5'-GGCGAtAAGGGtTACGTGC-3' 5'-AGCATCACTGTCCcGCATG-3'	[53]
18S rDNA	*CDLS* *CDLA*	5'-GCT CCA AAA GCG TAT ATT AAT GCT GT-3' 5'-TCC TTC ATT CCT AGA GGC CGT GAG T-3'	[52]
Not specified	*Pan081* *Bra1c y* *Guy901*	Not specified	[57]

Table 4. Comparative analysis of the Tm for each *Leishmania* spp. according to the target selected by different authors.

Species	Tm (℃)	Target	Equipment	Ref.
Leishmania braziliensis	79.69	*kDNA1*	Abi 7500	[47]
	83.08±0.21	*kDNA*	Rotor-Gene 6000	[36]
	77.34±0.01	*kDNA*	Abi 7500	[46]
	83.56±0.01	*kDNA*	Rotor-Gene 6000	[15]
	83.46±0.04	*kDNA*	Rotor-Gene 6000	[15]
	85.86±0.01 84.51±0.03	*hsp70*	PikoReal96	[18]
	90.01	*hsp70*	Abi 7500	[17]
	78.87	*ITS1*	Abi 7500	[17]
	76.80-77.90	*ITS1*	Abi 7500	[16]
	78.40-79.50	*ITS1*	Stratagene Mx3000P	[16]
	90.85±0.02	*Lack* gene 1	Light Cycler 480	[38]
	83.44±0.18	*Aap3 1*	PikoReal96	[39]
	85.97±0.04	*Aap3 3*	PikoReal96	[39]
	88.60±0.10	*Gp63*	ABi 7900	[49]
	74.06±0.10	*MPI*	LightCycler 480	[27]
	68.26±0.20	*6PGD*	LightCycler 480	[27]
	83.56±0.01	*Bra1C*	Rotor-Gene Q	[57]
	67.10	*18S rDNA*	Light Cycler 3.5	[52]
Leishmania guyanensis	79.41	*kDNA1*	Abi 7500	[47]
	83.21±0.27	*kDNA*	Rotor-Gene 6000	[36]
	77.90	*kDNA1*	Abi 7500	[51]
	77.39±0.01	*kDNA*	Abi 7500	[46]
	86.14±0.02 84.01±0.00	*hsp70*	PikoReal96	[18]
	90.03	*Hsp70*	Abi 7500	[17]
	79.23	*ITS1*	Abi 7500	[17]
	77.40-77.80	*ITS1*	Abi 7500	[16]
	78.60-79.20	*ITS1*	Stratagene Mx3000P	[16]
	83.83±0.15	*Aap3 1*	PikoReal96	[39]
	86.14±0.11	*Aap3 3*	PikoReal96	[39]
	71.76±0.10	*MPS*	LightCycler 480	[27]
	60.26±0.10	*6PGD*	LightCycler 480	[27]
	82.21	*Guy901*	Rotor-Gene Q (Qiagen).	[57]
Leishmania panamensis	79.99	*kDNA1*	Abi 7500	[47]
	83.07±0.09	*kDNA*	Rotor-Gene 6000	[36]
	78.70	*Kdna1*	Abi 7500	[51]
	89.74	*Hsp70*	Abi 7500	[17]
	77.00-77.90	*ITS1*	Abi 7500	[16]
	78.40-79.30	*ITS1*	Stratagene Mx3000P	[16]
	71.66±0.10	*MPI*	LightCycler 480	[27]
	56.26±0.70	*6PGD*	LightCycler 480	[27]
	86.55	*Pan081*	Rotor-Gene Q (Qiagen).	[57]
Leishmania shawi	79.78	*kDNA1*	Abi 7500	[47]
	78.50	*kDNA1*	Abi 7500	[51]
	77.34±0.01	*kDNA*	Abi 7500	[46]
	86.80±0.01* 84.07±0.01	*hsp70*	PikoReal96	[18]
	83.92	*Aap3 1*	PikoReal96	[39]
	86.20	*Aap3 3*	PikoReal96	[39]
Leishmania naiffi	80.88	*kDNA1*	Abi 7500	[47]
	80.70	*kDNA1*	Abi 7500	[51]
	77.34±0.01	*kDNA*	Abi 7500	[46]
Leishmania amazonensis	83.91±0.20	*kDNA*	Rotor-Gene 6000	[37]
	53.56±0.10	*MPI*	LightCycler 480	[27]
	89.37	*Hsp70*	Abi 7500	[17]
	81.13	*ITS1*	Abi 7500	[17]
	83.00	*Aap3 1*	PikoReal96	[39]
	84.43	*Aap3 2*	PikoReal96	[39]
	86.45	*Aap3 3*	PikoReal96	[39]
Leishmania donovani	78.00-79.00	*ITS1*	Abi 7500	[16]
	79.50-80.20	*ITS1*	Stratagene Mx3000P	[16]
	85.01±0.04	*hsp70*	PikoReal96	[18]
	68.00	*ITS1*	N.S.	[63]
	83.26	*kDNA a*	LightCycler	[45]
	87.12	*kDNA b*	LightCycler	[45]
	82.47±0.16	*Aap3 1*	PikoReal96	[39]
	85.23±0.30	*Aap3 2*	PikoReal96	[39]
	86.04±0.08	*Aap3 3*	PikoReal96	[39]
	88.50±0.40	*kDNA*	N.S.	[31]
	91.31±0.02	*Lack* gene 1	Light Cycler 480	[38]
	89.50±0.20	*Gp63*	ABi 7900	[49]
	71.70	18S rDNA	Light Cycler 3.5	[52]
	81.80	*Cpb*	iCycler	[53]
Leishmania infantum infantum	78.00-79.00	*ITS1*	Abi 7500	[16]
	79.50-80.20	*ITS1*	Stratagene Mx3000P	[16]
	65.00±0.20	*ITS1*	RotorGene	[32]
	83.20±0.47 84.32±0.34	*kDNA*	Rotor-Gene 6000	[36]
	89.40±0.30	*kDNA*	StepOne Plus	[35]
	78.95±0.01	*kDNA*	Abi 7500	[46]
	58.70	*MPI*	LightCycler 480	[27]
	90.58±0.02	*7SL*	RotorGene 6000	[33]
	83.26	*kDNA a*	LightCycler	[45]
	89.21-89.47	*kDNA b*	LightCycler	[45]
	90.50±0.20	*kDNA*	N.S.	[31]
	89.19	*Hsp70*	Abi 7500	[17]
	78.49	*ITS1*	Abi 7500	[17]
	82.51	*Aap3 1*	PikoReal96	[39]
	85.02	*Aap3 2*	PikoReal96	[39]
	85.67	*Aap3 3*	PikoReal96	[39]
	89.30±0.30	*kDNA*	LightCycler	[41]
	85.30	*kDNA*	RotorGene 6000	[62]
	80.10	*kDNA*	ABI Prism 7500	[48]
	84.30±0.04	*kDNA*	RotorGene 6000	[15]
	84.37±0.04	*kDNA*	RotorGene 6000	[15]
	84.44±0.36	*kDNA*	RotorGene 6000	[37]
Leishmania major	80.00-80.80	*ITS1*	Abi 7500	[16]
	81.80-82.90	*ITS1*	Stratagene Mx3000P	[16]
	57.40±0.20	*cbp*	Light Cycler 1.5	[25]
	85.23±0.03	*hsp70*	PikoReal96	[18]
	53.00±0.30	*ITS1*	RotorGene	[32]
	53.00	*ITS1*	N.S.	[63]
	91.30±0.70	Tryparedoxin peroxidase gene	RotorGene 3000	[24]

Table 4. Continued.

Species	Tm (℃)	Target	Equipment	Ref.
	86.58±0.11* 84.48±0.01	*hsp70*	PikoReal96	[18]
	83.89±0.26	*Aap3 1*	PikoReal96	[39]
	86.00±0.21	*Aap3 1*	PikoReal96	[39]
Leishmania lainsoni	78.96	*kDNA1*	Abi 7500	[47]
	77.90	*kDNA1*	Abi 7500	[51]
	77.37±0.01	*kDNA*	Abi 7500	[46]
	85.60±0.01 84.07±0.01	*hsp70*	PikoReal96	[18]
	84.39	*Aap3 1*	PikoReal96	[39]
	85.88	*Aap3 3*	PikoReal96	[39]
	67.86±0.20	*MPI*	LightCycler 480	[27]
	65.06±0.20	*6PGD*	LightCycler 480	[27]
Leishmania peruviana	70.16±0.30	*MPI*	LightCycler 480	[27]
	68.06±0.30	*6PGD*	LightCycler 480	[27]
Leishmania infantum chagasi	85.04±0.03	*hsp70*	PikoReal96	[18]
	78.00-79.00	*ITS1*	Abi 7500	[16]
	79.50-80.20	*ITS1*	Stratagene Mx3000P	[16]
	89.50±0.30	*Gp63*	ABi 7900	[49]
Leishmania mexicana	80.00–80.80	*ITS1*	Abi 7500	[16]
	81.10–81.60	*ITS1*	Stratagene Mx3000P	[16]
	85.46±0.06	*hsp70*	PikoReal96	[18]
	80.00	*kDNA1*	Abi 7500	[47]
	80.40	*kDNA1*	Abi 7500	[51]
	53.60	*MPI*	LightCycler 480	[27]
	88.83	*Hsp70*	Abi 7500	[17]
	89.40±0.10	*Gp63*	ABi 7900	[49]
	83.12±0.00	*Aap3 1*	PikoReal96	[39]
	84.46±0.05	*Aap3 2*	PikoReal96	[39]
	86.88±0.09	*Aap3 3*	PikoReal96	[39]
Leishmania amazonensis	80.20–80.40	*ITS1*	Abi 7500	[16]
	81.10–81.30	*ITS1*	Stratagene Mx3000P	[16]
	85.39±0.03	*hsp70*	PikoReal96	[18]
	80.37	*kDNA1*	Abi 7500	[47]
	84.09±0.26	*kDNA*	Rotor-Gene 6000	[36]
	80.10	*kDNA1*	Abi 7500	[51]
	78.94±0.00	*kDNA*	Abi 7500	[46]
Leishmania major	86.50±0.20	*kDNA*	StepOne Plus	[35]
	89.27±0.06	*7SL*	RotorGene 6000	[33]
	83.18	*kDNA a*	LightCycler	[45]
	83.86	*kDNA b*	LightCycler	[45]
	87.50±0.30	*kDNA*	N.S.	[31]
	89.40±0.10	*Gp63*	ABi 7900	[49]
	82.29±0.24	*Aap3 1*	PikoReal96	[39]
	85.90±0.11	*Aap3 2*	PikoReal96	[39]
	85.57±0.04	*Aap3 3*	PikoReal96	[39]
	88.30±0.03	*Lack* gene 1	Light Cycler 480	[38]
	87.00±0.50	*kDNA*	LightCycler	[41]
Leishmania tropica	79.00-79.50	*ITS1*	Abi 7500	[16]
	80.20-80.80	*ITS1*	Stratagene Mx3000P	[16]
	66.60±0.10 48.10±0.50 or 55.80±0.60	*cbp*	Light Cycler 1.5	[25]
	84.40±0.02	*hsp70*	PikoReal96	[18]
	61.00±0.20	*ITS1*	RotorGene	[32]
	62.00	*ITS1*	N.S.	[63]
	89.50±0.20	Tryparedoxin peroxidase gene	RotorGene 3000	[24]
	88.30±0.20	*kDNA*	StepOne Plus	[35]
	83.03	*kDNA a*	LightCycler	[45]
	88.89	*kDNA b*	LightCycler	[45]
	89.49±0.09	*7SL*	RotorGene 6000	[33]
	89.78±0.04	*7SL*	RotorGene 6000	[33]
	89.30±0.20	*kDNA*	N.S.	[31]
	82.22±0.02	*Aap3 1*	PikoReal96	[39]
	86.47±0.08	*Aap3 2*	PikoReal96	[39]
	85.91±0.07	*Aap3 3*	PikoReal96	[39]
	88.75±0.01	*Lack* gene 1	Light Cycler 480	[38]
	88.50±0.20	*kDNA*	LightCycler	[41]
	65.00	18S rDNA	Light Cycler 3.5	[52]
Leishmania aethiopica	80.60	*ITS1*	Abi 7500	[16]
	81.90-82.30	*ITS1*	Stratagene Mx3000P	[16]
	55.20±0.50	*cbp*	Light Cycler 1.5	[25]
	91.60±0.03	*Lack* gene 2	Light Cycler 480	[38]

*The authors analyzed two amplicons from the same target; the highlight represents the temperature of amplicon 1 and no highlight represents the temperature of amplicon 2.

equipment used in these references. The most explored target was kDNA that was used in 13 reports, with the primers pair JW13/JW14 being used in four of them[31,35,41,45].

Eleven references used only MCA for the diagnosis and/or identification of *Leishmania* spp. The first report to use MCA was published in 2002[45] and was able to distinguish among cultured strains based on their Tm peaks: *L. major* (83.18 ℃), *L. donovani* (87.12 ℃), *L. tropica* (88.89 ℃) and *L. infantum* (89.21 ℃). These latter two species were difficult to differentiate because the Tm difference between them was less than 1 ℃. The MCA targeted a fragment of the kDNA minicircle using the primers JW13/JW14, which were also employed in the next three references. De Mombrison *et al.*[31] correctly identified the parasite in 55% of the samples from human cutaneous lesions in Algeria based on pre-established Tm values for reference strains. The species identified were *L. major* [(87.5 ± 0.3) ℃], *L. donovani* [(88.5 ± 0.4) ℃], *L. tropica* (89.3 ± 0.2) ℃] and *L. infantum* [(90.3 ± 0.2) ℃]. A certain degree of overlapping (≤1 ℃) among the strains and a distinct peak [(84.4 ± 0.4) ℃] were detected in 24% of the samples in which the species could not be determined. Abda *et al.*[41] used

dermal scrapings collected from lesion borders of 27 patients in Tunisia and reported positive results/species identification in 81.5% of cases. Again, the Tm provided better discrimination between *L. major* [(87 ± 0.5) ℃] and *L. infantum* [(89.3 ± 0.3) ℃], although overlap with *L. tropica* [(88.5 ± 0.2) ℃] led to a couple of misidentifications, later resolved by MLEE as *L. tropica* killicki. As previously reported, a distinct Tm of (84.3 ± 0.3) ℃ was detected in four samples that were associated with *L. infantum*. In 2011, Khademvatan *et al.*, reported the analyses of 102 stained samples from CL patients in Iran, 94% of whom were positive by qPCR, with MCA identifying three species: *L. infantum* [(89.4 ± 0.3) ℃], *L. tropica* [(88.3 ± 0.2) ℃] and *L. major* [(86.5 ± 0.2) ℃], the Tm of which were compared to those of reference strains; the amplicons were also sequenced to confirm the species identification[35].

By targeting another fragment of kDNA minicircle (120 bp), Pita-Pereira *et al.* used MCA to identify the species that caused VL and CL in 30 Brazilian patients. The initial analysis of reference strains allowed classification of the species to the subgenera *Viannia* (77.34 ℃) and *Leishmania* (78.95 ℃), with the biopsies from CL patients being placed in the *Viannia* group and the peripheral blood and bone marrow aspirates from VL patients in the *Leishmania* group. The specimens had been previously diagnosed by microscopy, culture and/or PCR-RFLP[46]. Similarly, another study using kDNA analyzed blood samples from humans, cats, and dogs (n=300). A total of 223 samples were characterized into two ranges based on the Tm average obtained from the different strains of species tested. Thus, range 1 (78-79.99 ℃) contained 58 specimens that included species from the subgenus *Viannia*: *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. lainsoni* and *L. shawi*, and range 2 (80-82.2 ℃) contained 165 specimens from the subgenus *Leishmania*: *L. mexicana* and *L. amazonensis*, but also included *L. naiffi* from the subgenus *Viannia*. There was however, some disagreement between the results with MCA and other techniques such as MLEE (20%), PCR-RFLP (72.2%) and sequencing (56.4%), this discrepancy indicated the incorrect classification of some samples in the two established ranges[47]. MCA has also been used to target kDNA in urine from patients suspected of having VL, in which *L. infantum* was detected, thus confirming the results found in blood[48].

MCA has been used to target several other genes. In 2008, a study used the highly conserved *GP63* gene that encodes a surface metalloprotease, implicated in *Leishmania* virulence. The authors designed a pair of primers able to detect several species of reference strains by qPCR, but the MCA indicated similar Tm among them, *L. major* [(89.4 ± 0.1) ℃], *L. donovani* [(89.5 ± 0.2) ℃], *L. chagasi* [(89.5 ± 0.3) ℃], *L. mexicana* [(89.4 ± 0.1) ℃], and *L. braziliensis* [(88.6 ± 0.1) ℃] with the latter species differing from the others by a Tm difference ⩾1 ℃ from the others[49]. Khosravi *et al.*, analyzed 100 biopsies from CL patients in Iran. These authors targeted the tryparedoxin peroxidase gene (three copies) and performed a qPCR with MCA that yelded two peaks with the reference strains used being *L. major* [(91.3 ± 0.7) ℃] and *L. tropica* [(89.5 ± 0.2) ℃]. Seventy-five samples had *L. major* and five had *L. tropica*[24].

Another study based on MCA that targeted a 220-275 bp fragment of the *ITS1* rDNA used 11 different species from 33 World Health Organization and Centers for Disease Control and Prevention reference strains, all of which were classified on MLEE and *ITS2* sequencing. The species were classified in groups, as follows: G1 that included species from the subgenus *Viannia*: *L. braziliensis*, *L. guyanensis*, *L. panamensis*, G2A that included *L. donovani* and *L. infantum/L. chagasi*, G2B that consisted only *L. tropica*, and G3 that included *L. aethiopica*, *L. amazonensis*, *L. mexicana* and *L. major*. A total of 1 051 specimens (biopsies, peripheral blood, bone marrow and stained slides) were tested, with 45% being positive. The human internal control used to monitor inhibition yielded positive results in all the samples tested. The use of two different real-time PCR platforms demonstrated a variation of 1.0-2.1 ℃ among the established groups[50]. De Paiva-Cavalcante *et al.* used about six pairs of primers from conventional PCR and evaluated them in several qPCR assays that targeted kDNA, *ITS1* and *SSU* rDNA to preferentially detect *L. braziliensis* relative to other species in specimens from dogs, cats and skin scrapings from humans with CL. The results showed that kDNA was the best for detecting *L. braziliensis* (80 ℃) in 90% of the species, followed by *SSU* rDNA (85 ℃) in 82%[51], in contrast, the discriminatory power among the other species tested was low.

Several studies have used qPCR in conjunction with specific fluorescent probes for MCA based on probe-template Tm. Here we describe five reports that used a FRET/MCA combination to detect *Leishmania* species. The first one was published in 2003, just a year after the first report of MCA in leishmaniasis. The authors targeted the 18S rDNA gene to classify the species into three clinically relevant groups. They ran a qPCR with one pair of primers and two probes (*L. braziliensis* and *L. donovani* complexes) and evaluated the usefulness of the assay against 17 reference strains of several species and 12 human specimens (blood, bone marrow and biopsies). The FRET/MCA results showed no overlapping Tm among the three groups established (A, B, C) and all the samples were positive. Group A included *L. aethiopica*, *L. tropica*, *L. major* and *L. mexicana* (64.9-65.1 ℃), group B included *L. donovani* complex (71.5-71.8 ℃) and group C the *L. braziliensis* complex (66.5-67.6 ℃)[52]. Not long after this report, Quispe-Quintaya *et al.* described the use of a hemi-nested PCR followed by FRET/MCA, with the addition of three probes at the end of each of the nested reactions. The targets were two cysteine protein B (*CPB*) isogenes used to distinguish the species within the *L. donovani* complex (*L. donovani*, *L. infantum* and *L. archibaldi*). The authors tested reference strains of several species from different geographical locations, including *L. tropica*, *L. major*, *L. aethiopica* and clinical samples from humans and dogs diagnosed with VL. The combination of peaks detected in both reactions would reflect the species' geographic origin. Although the analyses were useful in excluding non-*L. donovani* complex species, the Tm was apparently unable to distinguish among species of the complex since different species were classified in the same group and the same species were classified in different groups[53].

Table 5. Characteristics of studies included in the analysis.

First author/ Year of publication	Country	Population	Molecular target	Methodology	Main objective	Outcome
Nicolas, 2002[45]	France	Humans, culture	*kDNA*	Real-time MCA	Differentiate four species of Old World *Leishmania*	Melting temperature was able to differentiate *Leishmania major* from *Leishmania donovani*, *Leishmania tropica* and *Leishmania infantum*
Schulz, 2003[52]	Germany	Humans, culture	18S rDNA	Real-time FRET	Detect, differentiate and quantify *Leishmania* organisms	Discrimination of three relevant groups: *Leishmania donovani* complex, *Leishmania brasiliensis* complex, other *Leishmania* spp.
Quispe-Tintaya, 2005[53]	Various	Humans, dogs, culture	*cpb* gene	Real-time FRET	Identification of species and intraspecies of *L.donovani* complex	The assay was able to distinguish *Leishmania donovani* from *Leishmania tropica*, *Leishmania major*, and *Leishmania aethiopica*; and identify 5 groups within *Leishmania donovani* complex
De Monbrison, 2007[31]	France, Algeria	Humans, culture	*kDNA*	Real-time MCA	Identification of *Leishmania* species causing cutaneous form of the disease	Identification of three *Leishmania* species causing CL from skin lesions spotted on filter paper: *Leishmania major*, *Leishmania donovani* and *Leishmania infantum*
Tupperwar, 2008[49]	India	Mice, culture	*GP63*	Real-time MCA	Quantification of *Leishmania* species and monitoring its systemic distribution	There was discreet overlapping among Tm of *Leishmania donovani*, *Leishmania major*, *Leishmania mexicana*, and *Leishmania chagasi*; Tm of *Leishmania braziliensis* was distinctly lower than *Leishmania donovani*
Nasereddin, 2010[33]	Israel	Humans, culture	7SL RNA	Real-time HRM	Diagnosis of Old World leishmaniasis using the assay to differentiate *Leishmania tropica*, *Leishmania major* and the *Leishmania donovani* complex	HRM typed all the strains and distinguished genotypes Ⅰ and Ⅵ of *Leishmania tropica* found in Israel and Palestinian Authority
Abda, 2011[41]	Tunisia, France	Humans, culture	*kDNA*	Real-time MCA	Evaluate advantages and limits of the assay in the identification of *Leishmania* species in a routine diagnostic laboratory use	The assay was able to differentiate *Leishmania major*, *Leishmania infantum* and *Leishmania tropica*; and was considered an alternative to identify CL species directly from clinical samples
Khadenvatam, 2011[35]	Iran	Humans	*kDNA*	Real-time MCA	Describe the assay for the diagnosis and direct identification of *Leishmania* species on Giemsa-stained slides without cultivation	The assay was able to differentiate *Leishmania major*, *Leishmania infantum* and *Leishmania tropica* from Giemsa-stained slides stored more than 3 years
Khosravi, 2012[24]	Iran	Humans, culture	Tryparedoxin peroxidase gene	Real-time MCA	Detection and identification of Old Wold cutaneous leishmaniasis	Differentiation of *Leishmania major* and *Leishmania tropica* by the assay which was considered with greater sensitivity compared to microscopy and culture
Pita-Pereira, 2012[46]	Brazil	Humans, sand flies, culture	*kDNA*	Real-time MCA	Use the assay to discriminate *Leishmania* subgenera and identify etiological agents of CL and VL	The samples tested showed melting profile according to reference strains of *Leishmania amazonensis* and *Leishmania infantum*
Toz, 2013[54]	Turkey	Humans, dogs, culture	*ITS1*	Real-time MCA	Design an assay based on *ITS1* region to diagnose all clinical forms of leishmaniasis directly in clinical samples from humans and dogs	The assay was able to differentiate reference strains of *Leishmania donovani* complex, *Leishmania tropica* and *Leishmania major*; and identified *Leishmania* species in 81.58% of samples analyzed
Cavalcanti, 2013[51]	Brazil, Italy	Humans, dogs, cats, culture	*kDNA*, *ITS1* and *rDNA*	Real-time MCA	Develop qPCR assays to detect *Leishmania* (*Viannia*) *brazilisensis* from animals and apply to humans with suspect of ACL infection	Among the targets included, the kDNA1 was the more specific, amplifiyng more than one *Leishmania* specie and allow distinction of different species by melting curve
Tsukayama, 2013[27]	Various	Humans, culture	*MPI*, *6PGD*	Real-time FRET	Identify five main species causing tegumentary leishmaniasis in the New World	The assay was able to simultaneously diagnose New World leishmaniasis and identify five species more prevalent in South America

Table 5. Continued.

First author/ Year of publication	Country	Population	Molecular target	Methodology	Main objective	Outcome
Mohammadiha, 2013[62]	Iran	Dogs, culture	*kDNA*	Real-time MCA	Assess the efficacy of sera for *Leishmania infantum* detection from symptomatic and asymptomatic dogs	The assay could differentiate between *Leishmania*-infected dogs and dogs with active leishmaniasis
Eroglu, 2014[32]	Turkey	Humans, culture	*ITS1*	Real-time HRM	Find out the most effective clinical samples and methods to be used in the diagnosis of CCL	Among the methods analyzed, real-time PCR was the most sensitive, able to identify *Leishmania* species in different samples
Ceccarelli, 2014[42]	Italy	Dogs, culture	*kDNA*	Real-time HRM	Detection and estimation of the *Leishmania* parasites in canine samples; discriminate *Leishmania* subgenera	The assay detected *Leishmania infantum* in canine samples and discrimitaned *Leishmania* (*Leishmania*) from *Leishmania (Viannia*)
Hernández, 2014[17]	Colombia	Humans, insects, mammals, culture	*HSP70, ITS1*	Real-time HRM	Evaluate the potencial of the genes *HSP70* and *ITS1* to discrimintae six *Leishamnia* species of New World	The subsequent amplification of genes *HSP70* and *ITS1* allowed the identification of *Leishmania mexicana*, *Leishmania infantum* (*chagasi*), *Leishmania amazonensis*, *Leishmania* panamensis, *Leishmania braziliensis* and *Leishmania guyanensis*
Pessoa-e-Silva, 2016[48]	Brazil	Humans, culture	*kDNA*	Real-time MCA	Evaluate the usefulness of urine for detection of *Leishmania infantumin* VL-infected patients	The assay showed a good negative predictive value for untreated suspected patients and indicate a potencial alternative to follow up the efficacy of therapeutic approaches
Nath-Chowdhury, 2016[25]	Canada, France, USA	Humans, culture	*cpb* gene	Real-time FRET	Differentiate the main Old World CL species in cultures parasited and biopsy specimens	Identification of three Old World *Leishmania* species (*Leishmania aethiopica*, *Leishmania major* and *Leishmania tropica*) from direct samples in a single step
Zampieri, 2016[18]	Brazil	Humans, mice, insects, culture	*HSP70*	Real-time HRM	Discrimination of seven Brazilian *Leishmania* species and three Eurasian and African species	The protocol described identified *Leishmania* species that are importante for majority of cases in the Brazil and Eurasia
De Morais, 2016[47]	Brazil	Humans, dogs, cats, culture	*kDNA*	Real-time MCA	Evaluate method viability for differentiation of *Leishmania* species in comparison with the classical technique	Although the technique was unable to identify parasites at species level, it was possible identify the parasites in two different ranges with close Tms
Ceccarelli, 2017[37]	Italy	Humans, dogs	*kDNA*	Real-time HRM	Investigate *kDNA* sequence among differente *Leishmania* species and differentiate *Leishmania infantum* from *Leishmania amazonensis*	Real-time HRM alone did not allow the discrimination between *Leishmania infantum* and *Leishmania amazonensis*, but when two quantitative tests were performed together, the distinction was possible
Almeida, 2017[16]	USA, Turkey	Humans, culture	*ITS1*	Real-time MCA	Apply the technique using genus-specific primers targeting *ITS1* in reference strains and human samples	The methodology was able to differentiate four groups of *Leishmania* parasites
Marin, 2017[57]	Colombia	Humans, culture	*HSP70*	Real-time MCA	Develop the methodology for diagnosis and typification of three CL species in Colombia	The three primers analyzed was able to identify each *Leishmania* specie
Kuang, 2017[38]	China	Culture	*Lack* gene	Real-time HRM	Analyze the usefulness of the *Lack* gene for differentiation of *Leishmania*	The combination of two primers discriminated six *Leishmania* reference strains
Müller, 2018[39]	Norway, Brazil	Humans, cats, insects, mice, culture	*Aap3*	Real-time HRM	Use of the *Aap3* coding sequence as target for differentiation of *Leishmania* spp.	The dissociation profile of three amplicons of this region allowed the discrimination of six species of *Leishmania* (*Leishmania)* and five species of *Leishmania* (*Viannia*)
Diotallevi, 2020[15]	Italy, Brazil	Humans, dogs, culture	*kDNA*	Real-time HRM	Validate previous work using human, canine samples and strains from a Brazilian region	Evaluation of HRM peaks and Cq values allowed the correct identification of subgenus (*Leishmania* or *Viannia*) and the species (*infantum* or *amazonensis*)

The *ITS1* was also used as a target by Toz *et al.*[54], who designed two probes to detect the *L. donovani* complex (*L. donovani* and *L. infantum*), *L. major* and *L. tropica*. The analyses involved cultured isolates previously identified by MLEE (n=51), reference strains (n=4) and specimens from humans and dogs with CL or VL (n=315). The TM obtained in the pilot study varied among the species: *L. major* (53 ℃), *L. tropica* (62 ℃) and *L. donovani* complex (68 ℃). Among the specimens tested, *L. infantum* (60.6%) and *L. tropica* (21%) were identified, in addition to a two-peak (Tm) profile detected in 18.5% of the samples. Sequencing results corroborated 98% of the 102 sequenced samples, while MLEE matched 94.1% of the 51 isolates tested.

Tsukayama *et al.* targeted two loci, with the single copy genes *MPI* and *6PGD* being able to discriminate among six New World species in 79.1% of the clinical samples tested[27]. Although the technique could distinguish *L. amazonensis* and *L. infantum* from others species, these two were indistinguishable from each other because of their identical Tm. A similar approach, in which the polymorphic and multi-copy *CPB* gene was targeted using a single reaction, was able to distinguish three Old World species of *Leishmania* based in comparison with reference strains and cultured promastigotes from previously diagnosed specimens; tests on seven human biopsies detected *L. major* and *L. tropica*[55]. The use of FRET/MCA probes increased the sensitivity in detecting differences in Tm among species or groups when compared with MCA alone, in which overlapping curves hampered the identification and distinction of a higher number of species.

The use of HRMA to diagnose leishmaniasis was first reported in 2010. As with MCA, the technique measures changes in the fluorescence intensity of the dsDNA intercalating dye (SYBR Green) during dissociation, enabling the detection of SNPs in the targeted sequence. Advances in software/analysis features have increased the sensitivity in distinguishing among very similar Tm.

Nasereddin and Jaffe[33] targeted 7SL RNA and used reference strains to identify *L. tropica* [(89.49 ± 0.09) ℃], *L. major* [(89.27 ± 0.06) ℃] and the *L. donovani* [(90.8 ± 0.02) ℃] complex. The same gene and apparently the same sequence were later targeted by Mohammadi *et al.*[56], who concluded that HRMA was cheaper and less time-consuming compared with other techniques, such as sequencing (7SL RNA) and PCR-RFLP (ITS), and showed equal sensitivity and specificity for *L. major* [(87.03 ± 0.03) ℃], *L. tropica* [(88.09 ± 0.35) ℃] and *L. infantum* [(88.86 ± 0.02) ℃].

The use of kDNA in HRMA was first explored by Ceccarelli *et al.*, who tested two reactions, only one of which, using primers MLF-MLR, was able to distinguish between the subgenera *Viannia* and *Leishmania* in reference strains and dog specimens[36]. The same group later reported several cases of *L. infantum* that showed two peaks, with others having an undetermined Tm because of the lack of reproducibility among replicates, as also seen in *L. amazonensis*[37]. To validate their previous work, Diottalevi *et al.*[15] used the same approach and applied two qPCR to Brazilian strains and specimens (n=47) from humans and dogs with VL or CL; the *Leishmania* spp. was identified in 78.7% of the cases. Two human samples had *L. infantum* and the subgenus *Viannia*; the others were positive for *L. infantum* (n=36) and *L. amazonensis* (n=1). In Turkey, Eroglu *et al.*[32] compared the sensitivity of different diagnostic methods for chronic CL. The use HRMA to target the ITS1 region yielded positive results in 63.4% of the cases with confirmed leishmaniasis; the species detected were *L. tropica* (54.5%), *L. major* (24.3%) and *L. infantum* (21.2%).

The first report to describe the use of the *HSP70* gene in combination with *ITS1* as a potential genotyping tool for *Leishmania* spp. used reference strains characterized by PCR-RFLP/MLEE. The strains were isolated from humans, sand flies, and marsupials (n=39) from different geographical areas of Colombia. The established algorithm was able to distinguish among six New World species[17]. Later, Zampieri *et al.* amplified two targets within the *HSP70* gene and, among the reference strains tested, the first reaction (amplicon 1) was able to distinguish *L. tropica*, from other groups based on a Tm difference of 0.3 ℃, while the second reaction (amplicon 2) placed five species in two different groups: *L. braziliensis*/*L. guyanensis* and *L. lainsoni*/*L. amazonensis*/*L. mexicana*[18]. In Colombia, Marin *et al.*, searched the *HSP70* gene of *L. panamensis*, *L. braziliensis* and *L. guyanensis* for targets that could be validated by HRMA using reference strains. Three pairs of primers were selected based on their specificity for each of the species of interest. The runs were done separately and resulted in distinct peaks. Thirty-three isolates from biopsies previously tested by PCR-RFLP were blindly tested by HRMA; 87.8% of these were positive, with agreement of 90.6%[57]. However, the reproducibility of this assay was compromised because the authors did not report the sequences of the primers used.

Kuang *et al.*[38] explored the lack gene as a target for HRMA since it encodes an antigenic protein expressed in amastigotes and promastigote stages. Six reference strains from the WHO isolated from different countries and ten isolates from China (human, sand fly, rodent and canine) were tested. Two distinct reactions were run, the first of which detected and distinguished among four species: *L. donovani* [(91.31± 0.02) ℃], *L. tropica* [(88.75±0.01)℃], *L. major* [(88.30±0.03) ℃] and *L. braziliensis* [(90.85±0.02) ℃], while the second distinguished between *L. turanica* [(90.75±0.02) ℃] and *L. braziliensis* [(91.60±0.03) ℃].

Miller *et al.* targeted a coding sequence of the two-copy gene for amino acid permease 3 (AAP3), which has been less explored in studies of leishmaniasis. Reference strains from the WHO and additional ones from cultured isolates were tested. Three pair of primers were designed and tested in distinct reactions. Based on the authors' analyses, who considered a Tm difference of ±0.25 ℃ as distinct, the first reaction classified strains of the subgenus *Leishmania* into three clusters and those of *Viannia* also into three clusters, with *L. lainsoni* and *L. braziliensis* being distinct from one another and from the other clusters. The second reaction yielded a similar profile for the subgenus *Leishmania*, but now distinguished *L. major* from *L. tropica* and these from the other two clusters. The third reaction was able to distinguish among some species that had been clustered together by the previous reaction, *e.g. L. amazonensis*

from *L. mexinana*, *L. donovani* from *L. infantum* and, as in the second reaction, *L. major* from *L. tropica*[39].

In summary, kDNA has been the main target used for *Leishmania* spp. identification, followed by *ITS1*. Most investigations have explored a single target, using only pair of primers, although 2-3 pairs have also been used to amplify different regions in the same target/gene. In addition, 2-3 targets/genes have been used in combination to resolve species grouped by Tm (Table 3). All the strategies used so far have been able to identify *Leishmania* spp. by their Tm, with the number of the species ranging from one to 11 species.

To overcome the difficulty of differentiating among species with highly similar Tm, several reports have used Tm ranges that grouped species within subgenera or grouped species from distinct subgenera based on a pre-established algorithm. Reference strains and/or cultured parasites were used to establish "reference Tm" that were later applied to the classification of results from a wide variety of specimens. Although the use of different reactions has not always resulted in the detection of more species, Müller *et al.* created an algorithm using three qPCR for the (*AAP3* gene) that was able to identify 11 species of *Leishmania* by HRMA, while Tsukayama *et al.* were able to distinguish six species using two reactions (FRET/MCA)[27,39].

The use of appropriate equipment to perform HRMA allows the detection of small differences in the melting curve, but distinct enough to separate species. The use of probes (FRET/MCA) provides greater sensitivity in distinguish among the Tm of species or groups when compared with MCA alone since the occurrence of overlapping curves in the latter assay can hamper the identification of a large number of species.

Many studies have sought to identify *Leishmania* spp. based on their geographical incidence and the clinical symptoms caused by certain species. Indeed, geographical variation in the clinical manifestations of leishmaniasis is an important epidemiological issue. For example, genetic variability has been reported for the *L. donovani* complex[58], for *L. infantum* and *L. tropica* in southern Turkey[59], and for *L. infantum* from humans and dogs in different regions of Brazil[60,61].

Although many investigators have described algorithms based on MCA/HRMA for the simple, rapid detection/discrimination of *Leishmania* spp., such algorithms are not that simple or reproducible since variation in the discriminatory power of the assay has been noted even when using the same target/primers and the same species. In addition, as shown by Almeida *et al*, the use of different qPCR platforms can also introduce variation in the melting curve temperatures[16]. Indeed, MCA/HRMA alone has often been unable to identify the parasites, with a need for confirmation by sequencing (59.2%) or cloning (25.9%) (Table 2).

In conclusion, although the use of MCA/HRM has limitations in reproducibility and specificity, it has been considered a good tool to distinguish among *Leishmania* species and assist in choosing the appropriate therapeutic approach. The reports discussed here show that this method can distinguish *Leishmania* at the subgenus level and possibly identify some species within each subgenus.

The wide range of methodological variations to each of the assays discussed here limited the use of comparative analyses tools in this analysis. These limitations could be overcome by a multicentric study, with participants from different countries using strains from distinct geographical areas to standardize the methods and ensure the quality and robustness of *Leishmania* identification. Such a coordinated effort would also be helpful in optimizing the techniques and algorithms involved.

Conflict of interest statement

The authors have no conflicts of interest with this work.

Authors' contributions

JJGF designed the study, performed the data acquisition. JJGF and FSN defined the paper's content and wrote the manuscript. JJGF, FSN and GEBM analyzed the data, assisted in content definition and manuscript review. GEBM approved the grand, EAA and SCBC approved the project and lead the research and infrastructure needed. All the authors analyzed the data, reviewed, edited and approved the manuscript draft.

References

[1] Pan American Health Organization. *Leishmaniasis*. [Online]. Avaliable from: https://www.paho.org/hq/dmdocuments/2017/2017-cha-leishmaniasis-factsheet-work.pdf. [Accessed on 4 March 2019].

[2] Pan American Health Organization. *Leishmaniasis*: *Epidemiological report of the Americas 2018*. [Online]. http://iris.paho.org/xmlui/bitstream/handle/123456789/34856/LeishReport6_eng.pdf?sequence=1&isAllowed=y. [Accessed on 24 April 2018].

[3] Sangenito LS, da Silva Santos V, d'Avila-Levy CM, Branquinha MH, Souza dos Santos AL, de Oliveira SSC. Leishmaniasis and chagas disease-neglected tropical diseases: Treatment updates. *Curr Top Med Chem* 2019; **19**(3): 174-177.

[4] World Health Organization. *Control of the leishmaniases: Report of a meeting of the WHO expert commitee on the control of leishmaniases. Geneva, 2010*. [Online]. Avaliable from: https://apps.who.int/iris/handle/10665/44412. [Accessed on 27 April 2020].

[5] Burza S, Croft SL, Boelaert M. Leishmaniasis. *Lancet* 2018; **392**(10151): 951-970.

[6] Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. *Lancet Infect Dis* 2007; **7**: 581-596.

[7] Espada CR, Ortiz PA, Shaw JJ, Barral AMP, Costa JML, Uliana SRB, et al. Identification of *Leishmania* (*Viannia*) species and clinical isolates of *Leishmania* (*Leishmania*) amazonensis from Brazil using PCR-RFLP of the heat-shock protein 70 gene reveals some unexpected observations. *Diagn Microbiol Infect Dis* 2018. doi: 10.1016/

j.diagmicrobio.2018.03.004.

[8] Montalvo AM, Fraga J, Monzote L, Montano I, De Doncker S, Dujardin JC, et al. Heat-shock protein 70 PCR-RFLP: A universal simple tool for *Leishmania* species discrimination in the New and Old World. *Parasitology* 2010; **137**: 1159-1168.

[9] Rioux J, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of *Leishmania*: Use of isoenzymes. Suggestions for a new classification. *Ann Parasit Hum Comp* 1990; **65**(3): 111-125.

[10] Tsokana C, Athanasiou L, Valiakos G, Spyrou V, Manolakou K, Billinis C. Molecular diagnosis of leishmaniasis, species identification and phylogenetic analysis. In: *Leishmaniasis–Trends in epidemiology, diagnosis and treatment*. Missouri: Missouri State University; 2014. doi: http://dx.doi.org/10.5772/57353.

[11] Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of dna melting curves during the polymerase chain reaction. *Anal Biochem* 1997; **245**: 154-160.

[12] Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: A closed-tube method for differentiating homozygotes and heterozygotes. *Clin Chem* 2003; **49**(3): 396-406.

[13] Wittwer CT. High-resolution DNA melting analysis: Advancements and limitations. *Hum Mutat* 2009; **30**(6): 857-859.

[14] de Pita-Pereira D, Cardoso M, Alves C, Brazil R, Britto C. Detection of natural infection in *Lutzomyia cruzi* and *Lutzomyia forattinii* (Diptera: Psychodidae: Phlebotominae) by *Leishmania infantum* chagasi in an endemic area of visceral leishmaniasis in Brazil using a PCR multiplex assay. *Acta Trop* 2008; **107**: 66-69.

[15]Diotallevi A, Buffi G, Ceccarelli M, Neitzke-Abreu HC, Gnutzmann LV, da Costa Lima MS, et al. Real-time PCR to differentiate among *Leishmania* (*Viannia*) subgenus, *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis*: Application on Brazilian clinical samples. *Acta Trop* 2020; **201**: 105178.

[16]De Almeida ME, Koru O, Steurer F, Herwaldt BL, Da Silva AJ. Detection and differentiation of *Leishmania* spp. in clinical specimens by use of a *SYBR* green-based real-time PCR assay. *J Clin Microb* 2017; **55**(1): 281-290.

[17]Hernández C, Alvarez C, González C, Ayala MS, León CM, Ramírez JD. Identification of six New World *Leishmania* species through the implementation of a High-Resolution Melting (HRM) genotyping assay. *Parasit & Vectors* 2014; **7**(501): 1-7.

[18]Zampieri RA, Laranjeira-Silva MF, Muxel SM, Stocco de Lima AC, Shaw JJ, Floeter-Winter LM. High resolution melting analysis targeting *hsp70* as a fast and efficient method for the discrimination of *Leishmania* species. *PLoS Negl Trop Dis* 2016; **10**(2): 1-18.

[19]Tong SYC, Giffard PM. Microbiological applications of high-resolution melting analysis. *J Clin Microbiol* 2012; **50**(11): 3418-3421.

[20]Mohammad Rahimi H, Pourhosseingholi MA, Yadegar A, Mirjalali H, Zali MR. High-resolution melt curve analysis: A real-time based multipurpose approach for diagnosis and epidemiological investigations of parasitic infections. *Comp Immunol, Microbiol & Infect Dis* 2019; **67**: 101364. doi: 10.1016/j.cimid.2019.101364.

[21]De Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, Da Silva AJ. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. *J Clin Microbiol* 2011; **49**(9): 3143-3149.

[22]Folgueira C, Requena JM. A postgenomic view of the heat shock proteins in kinetoplastids. *FEMS Microbiol Rev* 2007; **31**(4): 359-377.

[23]Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. *Infect Genet Evol* 2010; **10**(2): 238-245.

[24]Khosravi S, Hejazi SH, Hashemzadeh M, Eslami G, Darani HY. Molecular diagnosis of old world leishmaniasis: Real-time PCR based on tryparedoxin peroxidase gene for the detection and identification of *Leishmania* spp. *J Vector Borne Dis* 2012; **49**(1): 15-18.

[25]Nath-Chowdhury M, Sangaralingam M, Bastien P, Ravel C, Pratlong F, Mendez J, et al. Real-time PCR using FRET technology for Old World cutaneous leishmaniasis species differentiation. *Parasit & Vectors* 2016; **9**(255): 1-11.

[26]Kuru T, Janusz N, Gadisa E, Gedamu L, Aseffa A. *Leishmania aethiopica*: Development of specific and sensitive PCR diagnostic test. *Exp Parasitol* 2011; **128**(4): 391-395.

[27]Tsukayama P, Núñez JH, De M, Santos L, Soberó NV, Lucas CM, et al. A FRET-based real-time PCR assay to identify the main causal agents of New World tegumentary leishmaniasis. *PLoS Negl Trop Dis* 2013; **7**(1): e1956.

[28]Tsukayama P, Lucas C, Bacon DJ. Typing of four genetic loci discriminates among closely related species of New World *Leishmania*. *Int J Parasitol* 2009; **39**(3): 355-362.

[29]Antonia AL, Wang L, Ko DC. A real-time PCR assay for quantification of parasite burden in murine models of leishmaniasis. *Peer J* 2018; **6**: e5905.

[30]Tricco AC, Lillie E, Zarin W, O'Brien KK, Colquhoun H, Levac D, et al. PRISMA extension for scoping reviews (PRISMA-ScR): Checklist and explanation. *Ann Intern Med* 2018; **169**(7): 467-473.

[31]De Monbrison F, Mihoubi I, Picot S. Real-time PCR assay for the identification of cutaneous *Leishmania* parasite species in Constantine region of Algeria. *Acta Trop* 2007; **102**: 79-83.

[32]Eroglu F, Uzun S, Koltas IS. Comparison of clinical samples and methods in chronic cutaneous leishmaniasis. *Am J Trop Med Hyg* 2014; **91**(5): 895-900.

[33]Nasereddin A, Jaffe C. Rapid diagnosis of Old World leishmaniasis by high-resolution melting analysis of the 7SL RNA gene. *J Clin Microbiol* 2010; **48**(6): 2240-2242.

[34]Nicolas L, Prina E, Lang T, Milon G. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. *J Clin Microbiol* 2002; **40**(5): 1666-1669.

[35]Khademvatan S, Neisi N, Maraghi S, Saki J. Diagnosis and identification of *Leishmania* spp. from Giemsa-stained slides, by real-time PCR and melting curve analysis in south-west of Iran. *Ann Trop Med Parasitol* 2011; **105**(8): 559-565.

[36]Ceccarelli M, Galluzzi L, Migliazzo A, Magnani M. Detection and characterization of *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by *SYBR* green-based real-time PCR and high resolution melt analysis targeting kinetoplast minicircle DNA. *PLoS One* 2014; **9**(2): e88845.

[37]Ceccarelli M, Galluzzi L, Diotallevi A, Andreoni F, Fowler H, Petersen C, et al. The use of kDNA minicircle subclass relative abundance to differentiate between *Leishmania* (*L.*) *infantum* and *Leishmania* (*L.*) *amazonensis*. *Parasit & Vectors* 2017; **10**(239): 1-10.

[38]Kuang Z, Zhang C, Pang H, Ma Y. A rapid high-resolution melting method for differentiation of *Leishmania* species targeting *lack* gene. *Acta Trop* 2017; **178**: 103-106.

[39]Müller KE, Zampieri RA, Aoki JI, Muxel SM, Nerland AH, Floeter-Winter LM. Amino acid permease 3 (aap3) coding sequence as a target for *Leishmania* identification and diagnosis of leishmaniases using high resolution melting analysis. *Parasit & Vectors* 2018; **11**(1): 421. doi: 10.1186/s13071-018-2989-z.

[40]Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**(4): 611-622.

[41]Abda I, De Monbrison F, Bousslimi N, Aoun K, Bouratbine A, Picot S. Advantages and limits of real-time PCR assay and PCR-restriction fragment length polymorphism for the identification of cutaneous *Leishmania* species in Tunisia. *Trans R Soc Trop Med Hyg* 2011; **105**(1): 17-22.

[42]Ceccarelli M, Galluzzi L, Sisti D, Bianchi B, Magnani M. Application of qPCR in conjunctival swab samples for the evaluation of canine leishmaniasis in borderline cases or disease relapse and correlation with clinical parameters. *Parasit & Vectors* 2014; **7**(460): PMID 25331737. doi:10.1186/s13071-014-0460-3.

[43]Vexenat A, Santana J, Teixeira R. Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania* (*Viannia*) *braziliensis*. *Rev Inst Med Trop S PAulo* 1996; **38**(3): 177-185.

[44]Caballero ZC, Sousa OE, Marques WP, Saez-Alquezar A, Umezawa ES. Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp. *Clin VACCINE Immunol* 2007; **14**(8): 1045-1049.

[45]Nicolas L, Milon G, Prina E. Rapid differentiation of Old World *Leishmania* species by LightCycler polymerase chain reaction and melting curve analysis. *J Microb Methods* 2002; **51**: 295-299.

[46]Pita-Pereira D, Lins R, Oliveira MP, Lima RB, Pereira BA, Moreira OC, et al. *SYBR* green-based real-time PCR targeting kinetoplast DNA can be used to discriminate between the main etiologic agents of Brazilian cutaneous and visceral leishmaniases. *Parasit & Vectors* 2012; **5**: 15.

[47]de Morais RCS, da Costa Oliveira CN, de Albuquerque S da CG, Mendonça Trajano Silva LA, Pessoa-e-Silva R, Alves da Cruz HL, et al. Real-time PCR for *Leishmania* species identification: Evaluation and comparison with classical techniques. *Exp Parasitol* 2016; **165**: 43-50.

[48]Pessoa-e-Silva R, Mendonça Trajano-Silva LA, Lopes da Silva MA, da Cunha Gonçalves-de-Albuquerque S, de Goes TC, Silva de Morais RC, et al. Evaluation of urine for *Leishmania infantum* DNA detection by real-time quantitative PCR. *J Microbiol Methods* 2016; **131**: 34-41.

[49]Tupperwar N, Vineeth V, Rath S, Vaidya T. Development of a real-time polymerase chain reaction assay for the quantification of *Leishmania* species and the monitoring of systemic distribution of the pathogen. *Diagn Microbiol Infect* Dis 2008; **61**: 23-30.

[50]de Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, da Silva AJ. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. *J Clin Microbiol* 2011; **49**(9): 3143-3149.

[51]de Paiva Cavalcanti M, Dantas-Torres F, da Cunha Gonçalves de Albuquerque S, Carla Silva de Morais R, Edileuza Felinto de Brito M, Otranto D, et al. Quantitative real time PCR assays for the detection of *Leishmania* (*Viannia*) *braziliensis* in animals and humans. *Mol Cell Probes* 2013; **27**: 122-128.

[52]Schulz A, Mellenthin K, Schönian G, Fleischer B, Drosten C. Detection, differentiation, and quantitation of pathogenic *Leishmania* organisms by a fluorescence resonance energy transfer-based real-time PCR assay. *J Clin Microbiol* 2003; **41**(4): 1529-1535.

[53]Quispe-Tintaya K, Laurent T, Decuypere S, Hide M, Bãnuls A, Doncker S, et al. Fluorogenic assay for molecular typing of the *Leishmania donovani* complex: Taxonomic and clinical applications. *Brazilian J Infect Dis* 2005; **192**: 685-692.

[54]Toz SO, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarli AT, et al. A real-time ITS1-PCR based method in the diagnosis and species identification of *Leishmania* parasite from human and dog clinical samples in Turkey. *PLoS Negl Trop Dis* 2013; **7**(5): e2205.

[55]Nath-Chowdhury M, Sangaralingam M, Bastien P, Ravel C, Pratlong F, Mendez J, et al. Real-time PCR using FRET technology for Old World cutaneous leishmaniasis species differentiation. *Parasit & Vectors* 2016; **9**(255): 1-11.

[56]Mohammadi MA, Bamorovat M, Harandi MF, Karimi T, Sharifi I, Aflatoonian MR. Comparison of three PCR-based methods for simplicity and cost effectiveness identification of cutaneous leishmaniasis due to *Leishmania tropica*. *Iran J Parasitol* 2017; **12**(2): 215-223.

[57]Marin J, Urrea D, Muskus C, Echeverry MC, Mejía AM, Triana O. Curvas de fusión de regiones genómicas específicas: Una herramienta prometedora para el diagnóstico y tipificación de las especies causantes de la leishmaniasis cutánea en Colombia. *Biomedica* 2017; **37**(4): 1-35.

[58]Franssen SU, Durrant C, Stark O, Moser B, Downing T, Imamura H, et al. Global genome diversity of the *Leishmania donovani* complex. *Elife* 2020; **9**: e51243.

[59]Eroglu F, Koltas IS, Alabaz D, Uzun S, Karakas M. Clinical manifestations and genetic variation of *Leishmania infantum* and *Leishmania tropica* in Southern Turkey. *Exp Parasitol* 2015; **154**: 67-74.

[60]Segatto M, Ribeiro LS, Costa DL, Costa CHN, de Oliveira MR, Carvalho SFG, et al. Genetic diversity of *Leishmania infantum* field populations from Brazil. *Mem Inst Oswaldo Cruz* 2012; **107**(1): 39-47.

[61]Santos Carvalho F, Rêgo Albuquerque G, Luiz Souza Carneiro P, Arias Wenceslau A. Genetic variability of *Leishmania infantum* in naturally infected dogs in the state of Bahia, Brazil. *Braz J Vet Parasitol* 2017; **26**(3): 389-394.

[62]Mohammadiha A, Haghighi A, Mohebali M, Mahdian R, Abadi AR, Zarei Z, et al. Canine visceral leishmaniasis: A comparative study of real-time PCR, conventional PCR, and direct agglutination on sera for the detection of *Leishmania infantum* infection. *Vet Parasito*l 2013; **192**(1-3): 83-90.

[63]Ozensoy Toz S, Culha G, Zeyrek YY, Ertabaklar F, Alkan H. A real-time ITS1-PCR based method in the diagnosis and species identification of *Leishmania* parasite from human and dog clinical samples in Turkey. *PLoS Negl Trop Dis* 2013; **7**(5): e2205.