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Molecular Diagnosis of Leishmaniasis, Species Identification and Phylogenetic Analysis

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1. Introduction

Leishmaniases are vector-borne infections caused by protozoa of genus *Leishmania*, affecting various mammals, mainly carnivores and humans. Clinical patent disease is relatively easy to be diagnosed and laboratory-confirmed by direct detection of the parasite in clinical samples. However, in subclinical cases detection of the causative agent is possible by highly sensitive diagnostic techniques such as molecular assays. Different molecular methods have been developed and evaluated including multilocus enzyme electrophoresis, conventional polymerase chain reaction (PCR) based assays, quantitative Real Time PCR as well as simplified PCR methods.

More than 30 *Leishmania* species have been recognized, of which 20 are considered infective for humans and animals. The ability to distinguish between *Leishmania* species is crucial for differentiation of various forms of disease (visceral, cutaneous, mucocutaneus) at least in humans in order to establish correct diagnosis and prognosis of the disease as well as to support decision-making regarding application of the appropriate treatment protocols.

Available tools for species identification and phylogenetic analysis include DNA sequencing analysis, restriction fragment length polymorphism (RFLP) analysis, and PCR-fingerprinting techniques as well as novel methods such as multilocus sequence typing (MLST) and multilocus microsatellite typing (MLMT). MLST is regarded as the most powerful phylogenetic approach and will be a better alternative to Multilocus Enzyme Electrophoresis (MLEE) in the future. Various studies showed that the same target genomic regions can be used to compare distances among species but also to evaluate genetic diversity within species.

This review aims to critically present current molecular approaches for leishmaniasis diagnosis, species identification and phylogenetic analysis.

2. Molecular diagnosis

PCR is being used for the diagnosis of parasitic diseases, including leishmaniasis. PCR is considered to be the most sensitive and specific technique among the methods applied so far for the direct detection and identification of the causative agent. The procedure is rapid and can be applied to a variety of clinical samples. Regarding the efficacy of the assay, it depends on the target selected for amplification (conserved or variable target region), the number of the target copies, the extraction technique used, the biological sample tested and the PCR protocol adapted or developed [1,2].

The PCR-based assays are advantageous over immunological techniques such as enzyme linked immunosorbent assay (ELISA) and immunofluorescence antibody test (IFAT) as host species specific reagents are not required. The increased PCR sensitivity over serology for the detection of infection is of great interest in certain cases such as in patients with cutaneous, muco-cutaneous leishmaniasis (CL or MCL) and the immunocompromised ones (e.g. coinfected with HIV, under chemotherapy etc). The former have low or no concentrations of antibodies against *Leishmania* due to the localized character of the disease while the latter present limited antibody production both resulting in negative serological tests [3]. In particular, in chronic CL patients, who constitute the greater diagnostic challenge due to their low parasite density, PCR assays for the detection of *Leishmania* DNA presented 100% sensitivity. Moreover, the fact that antibodies remain detectable for years after successful treatment makes the application of PCR a necessity[4].

PCR has been also proved to be valuable in the diagnosis of post-kala-azar dermal leishmaniasis (PKDL) [5]. Additionally, the detection of parasite DNA has been shown to be a useful prognostic marker for the disease relapse or the development of PKDL even after successful treatment outcome. [6]. Furthermore, persistent infection has been found in apparently healed scars from MCL patients [7], the presence of *Leishmania braziliensis* was reported in patients previously treated by immunotherapy or patients being at different stages of treatment and in subjects who had never presented clinical manifestations but they had lived in endemic areas and migrated to nonendemic regions [8].

Moreover, several studies reported that PCR detects parasitaemia a few weeks before the appearance of clinical manifestations. The detection of asymptomatic infected humans contributes to the prevention of the sand fly infection and the transfusion-transmitted kala-azar especially for the patients that require multiple transfusions, at least in endemic areas [3,9].

Regarding canine leishmaniasis, PCR assays constitute useful tools in cases of clinically healthy dogs which harbour infection but may never develop clinical disease. As the PCR positive results indicate infection, these assays could contribute to the prevention of the importation of infected clinically healthy dogs to nonendemic areas where infection may spread via local sand fly vectors and the transmission via blood transfusion [10]. Finally, the parasite detection is crucial in case of negative results obtained by serology. This discrepancy may be attributed

to the gap between infection and seroconversion, the transient presence of specific antibodies and the possibility for some infected dogs never to be seroconverted. In contrast, false positive results may be obtained due to the existence of anti-*Leishmania* antibodies for a considerable time after convalescence [11]. On the other hand, a positive PCR result in asymptomatic dogs cannot support decision-making regarding treatment as the parasite DNA may be present for a long time after the parasite has been cleared while also a single negative PCR result in a clinically suspected dog cannot rule out infection. Along with the need for PCR assays simplification, there is also a demand for standardization and optimization due to the lack of a universal PCR assay for the diagnosis of leishmaniasis [12]. Most laboratories perform "in-house" PCR assays using different primer pairs, DNA targets and PCR protocols [13].

A variety of clinical samples have been used for the detection of *Leishmania* DNA such as whole blood, buffy coat, bone marrow, lymph node, spleen, conjunctival swabs [14,15] and other biological samples such liver, lung, heart, penis, vagina, testis, semen, uterus, placenta, kidney, intestine, milk and urine [16] and more recently nasal, ear and oral swabs [17,18]. Bone marrow, lymph node, spleen and skin are the tissues presenting the highest sensitivity for the diagnosis of canine leishmaniasis [11,19]. The same holds true for the non invasive sampling techniques using conjunctival swabs [15,17]. Whole blood, buffy coat, urine and the other biological samples mentioned above have been shown to be less sensitive.

Several target sequences and different PCR protocols have been described for the detection of *Leishmania* DNA. The most frequently used amplification targets are the Kinetoplast DNA minicircle (kDNA) [20–25] and the small subunit ribosomal RNA (SSU rRNA) [26–29]. There are various gene targets which are also commonly used such as the ribosomal internal transcriped spacer (ITS) [15,30–34], the mini-exon gene (spliced leader) [32,35–40] and a repetitive genomic sequence [41,42].

It is worth mentioning that variable and sometimes conflicting results have been reported by several studies evaluating PCR using different target sequences in different host tissues. These results have been mostly obtained from asymptomatic infected hosts and they may vary depending on the sampling technique, storage method and the PCR protocol employed [1]. Some indicative studies evaluating the most frequently used PCR targets in different tissues are summarized in Table 1.

Target	PCR product size (bp)	Tissue tested	Sensitivity %	Specificity %	References
kDNA	120,297,790,792	WB,BM	68.8-100	100	[21] [22] [24] [25]
ssurRNA	358, 603	WB,BM	72.2-97	100	[27] [43][29] [28]
ITS1	300-350	BM,WB,SB,SS,DS,CS,CB,SA	68-100	100	[44] [33] [30] [32] [31]
Mini-exon	378-450	BM,WB,SB,LA,DB,GB	53.8-89.7	100	[37] [38] [35] [32]

BM: Bone marrow, WB: whole blood, SB: Skin biopsy, SS: Skin scrapings, DS: Dermal smear, SA: Skin aspirates LA: Lesion aspirates CS: Conjunctival swab, CB: Cultured biopsies, DB: Duodenal biopsy, GB: Gastric biopsy

Table 1. Evaluation of the most frequently used PCR targets in different tissues

Real time PCR (or quantitative PCR-qPCR), a molecular technique which has revolutionized the pathogen diagnosis, is considered to be the future reference method for molecular diagnosis. In recent years, qPCR assays based either on SYBR Green or TaqMan chemistries have been developed and evaluated for the detection, quantification and even species differentiation of *Leishmania spp* in a variety of clinical samples showing high sensitivity and reproducibility [45,46]. qPCR is considered to be a helpful tool for *Leishmania* diagnosis, monitoring during therapy, development of new drugs and diagnostic tools, comparison of drug efficacy or prophylactic schemes, and for epidemiological studies. Regarding diagnosis of leishmaniasis, the kinetic study of parasitemia in the immunocompromised hosts, the diagnosis of relapses and the quantification of the low parasitic load in asymptomatic patients are of great interest [47].

qPCR is highly sensitive especially at the lower parasite loads [48,49], specific and reproducible offering the ability to monitor therapy and to prevent relapses. The applications mentioned above make qPCR an attractive alternative to conventional PCR in routine diagnosis [47,49]. Some of the studies carried out so far and their findings regarding the detection threshold, sensitivity and specificity are summarized in Table 2.

Target	Tissue tested	Detection threshold	Sensitivity %	Specificity %	References
kDNA	BM, WB	0.001 p/ r			[50]
kDNA	WB	0.07 p/ r	100	83.33	[51]
kDNA	BM, WB, LN, CS, S, L, LU,K, BC	0.03 p/ r			[52]
kDNA	WB	0.004 p/ r			[53]
TRYP	BS		98.7	59.8	[54]
ITS1	WB, SB, S	0.25 p/s			[55]

BM: Bone marrow, WB: whole blood, SB: Skin biopsy, CS: Conjunctival swab, LN: Lymph node, S: Spleen, L: Liver, LU: Lung, K: Kidney, BC: Buffy coat, BS: Biopsy specimen, p/r:parasites/reaction, p/s: parasite/sample TRYP: tryparedoxin peroxidase gene

Table 2. Detection threshold, sensitivity and specificity of qPCR using various targets in different tissues

Given that PCR is restricted to well equiped laboratory settings, and that there is a need for simplification of the PCR assay and a demand for standardization and optimization [56], the described tools below may represent a good alternative for rapid and simple diagnosis of leishmaniasis in endemic areas and epidemiological studies [12,57].

Quantitative nucleic acid sequence-based amplification (QT-NASBA) has proven to be a very sensitive and specific assay in diagnostic microbiology which is based on the amplification of single-stranded RNA sequences. In fact, this technique detects RNA in a background of DNA [13]. Several QT-NASBA assays have been developed for the detection of *Leishmania* parasites including QT-NASBA combined with electro-chemiluminescence (ECL) [57,58] and QT-NASBA combined with oligochromatographic technology (OC) [12,59] for the detection of NASBA products. The QT-NASBA assays developed, are commonly based on amplification

of single-stranded 18S ribosomal RNA sequences [12,57,58,60,61]. This target is considered to be highly efficient for the diagnosis of leishmaniasis as each parasite contains a large number of copies of the 18SrRNA gene [62] while also the cytoplasm is assumed to contain approximately 104 rRNA copies [62]. Moreover the target is present in all *Leishmania* species and it does not vary between different species allowing high sensitivity and quantification of all species in a similar manner [12,57,58]. However, this target shows high similarity with the 18S rRNA gene sequence of *Endotrypanum, Crithidia, Wallaceina*, and *Leptomonas* organisms which may result in false positive results especially in the case of immunocompromised patients [12]. The fact that NASBA detects RNA, makes it a molecular tool of great importance for the measurement of viable parasites. As a consequence, its application makes possible the assessment of the efficacy of drug therapies, the prediction of treatment outcome and the monitoring of the emergence of drug resistance. As it is well known, the DNA is still detected for a long time after parasite death, thus making RNA a preferable amplification target for the demonstration of parasite viability [13,56,58]. Moreover, when targeting RNA, the starting number of the template molecules is much higher resulting in increased assay sensitivity and decreased sample volume required [56]. The latter, makes also QT-NASBA a highly sensitive assay as it is able to detect very low target levels on clinical samples.

Loop-mediated isothermal amplification (LAMP), a novel method of DNA amplification under isothermal condition [63], has been developed to detect *Trypanosoma spp, Plasmodium spp, Mycobacterium spp and Filaria spp* [64]. Recently a reverse transcriptase step has been developed to specifically amplify RNA so as to amplify RNA viruses such as HIV and avian influenza viruses and to increase the assay sensitivity [65]. The recently developed LAMP seems to be a promising diagnostic tool. The results obtained from several studies are encouraging as this assay is much faster than conventional or nested PCR, it may be applied in field conditions, it shows high specificity and sensitivity [63,64,66–69].

In the context of a generalized effort for simplification of the parasite detection, assays including PCR-ELISA and PCR-OC have been developed and evaluated. Several studies reported that PCR-ELISA showed high sensitivity. In a study, PCR-ELISA in blood samples from HIV negative VL patients was evaluated and presented higher sensitivity (83.9% and 73.2%) and specificity (100% and 87.2%) than conventional PCR [70]. Other investigators have also evaluated the use of the assay in blood samples from HIV co-infected VL patients and PCR-ELISA found to be highly sensitive [23,71,72]. Basiye et al, reported that PCR-OC is highly sensitive for *Leishmania* diagnosis on blood samples from VL patients (sensitivity 96,4% and specificity 88.8%) compared to NASBA-OC which was shown to be more specific (specificity 100%) [60]. In another study the repeatability and reproducibility of the assay was studied and found to be 95.9% and 98.1% in purified nucleic acid specimens and 87.1% and 91.7% in blood specimens spiked with parasites respectively [73].

3. Species identification

The species identification is useful in areas with various sympatric *Leishmania* species such as the southern Mediterranean Basin where CL is caused by *L. major*, *L. tropica* or *L.infantum* and South America where CL may be caused by *L.mexicana* and *L.amazonensis* as well as the species

of the subgenus *L.(Viannia)*. Regarding the areas where only one species is considered to be responsible for the disease, the species identification is an important tool for the differentiation between *Leishmania* species and lower trypanosomatids related to the monoxenous parasites of insects of the genera *Leptomonas* or *Herpetomonas* which are also considered to cause VL in Southern Europe, South America and in the Indian subcontinent. As far as it concerns the non-endemic areas, they seem to be at risk for parasite importation due to the increasing international travel and population migration [74].

In recent years, there has been great scientific interest in the development of molecular tools, based on PCR or other amplification techniques, for *Leishmania* parasites identification at species and even strain level. The molecular tools used, range from amplification and subsequent RFLP or DNA sequence analysis of multicopy targets or multigene families, including coding and non-coding regions, and PCR-fingerprinting techniques to the recently developed MLST and MLMT with different discriminatory power, sensitivity and specificity while also each one has its specific advantages and drawbacks [74]. Additionally, in most cases, the level of polymorphism found with coding or repeated non-coding PCR-amplified sequences is not refined enough to distinguish between closely related strains while application of MLST and MLMT approaches may reveal important strain polymorphisms.

PCR assays amplifying the conserved region of kinetoplast minicircle DNA or SSU rDNA have been shown to be the most sensitive, but they are able to identify *Leishmania* parasites only to the generic and/or subgeneric level [34,35,41,62]. However, the kDNA PCR-RFLP assay has been used as a molecular marker for *Leishmania* identification at strain level and found to be discriminative between closely related organisms such as *L.infantum* MON-1. In this case, PCR-RFLP of whole minicircle DNA, a highly polymorphic assay, has been applied for differentiation between recrudescence and re-infection [75,76] and for *L.infantum* strain typing [77]. However, the interpretation of the RFLP patterns is difficult as well as the comparison of the results obtained between laboratories [74,77].

The targets used for species identification include the ribosomal internal transcribed spacer (ITS) [34,78,79]; the mini-exon gene [38,39]; repetitive nuclear DNA sequences [80]; the glucose-6-phosphate dehydrogenase gene [81]; gp63 genes [82]; hsp70 genes[83,84]; cytochrome b gene [85], 7SL RNA gene sequences [86].

Other PCR-based approaches used for *Leishmania* parasites identification at strain level include the sequences of cysteine protease B (cpb) gene [87–90], the gp63 [87,91], the ITS1 [33,92–94], the mini-exon [95] and the kinetoplast minicircles [76,96–99].

The digestion of ITS1 PCR product with the restriction enzyme HaeIII can distinguish all medically relevant *Leishmania* species. However, almost identical RFLP patterns arise for the representatives of the *L. donovani* complex *(L. donovani* and *L.infantum)* or *L. braziliensis* complex *(L. braziliensis, L. guyanensis, L. panamensis, L. peruviana* etc.) with a great variety of restriction enzymes [34]. According to Schönian et al, in such a case, the sequencing of the ITS1 PCR product will allow the species differentiation [74]. Nasereddin et al developed a simple reverse line blot hybridization (RLB) assay based on ITS1 sequences, which could distinguish all Old World *Leishmania* species, even *L. donovani* from *L.infantum*. This approach was found to be

highly sensitive, approximately 10- to100-fold more sensitive than ITS1 PCR while the results obtained were comparable to those found by kDNA PCR [79]. Moreover, Talmi-Frank et al, described a new application of high resolution melt (HRM) analysis of a real time PCR product from the ITS1 region in samples from human, reservoir hosts and sand flies for rapid detection, quantification and speciation of Old World *Leishmania* species. In this assay, different characteristic high resolution melt analysis patterns were exhibited by *L.major*, *L.tropica*, *L. aethiopica*, and *L.infantum* making this approach able to distinguish all Old World *Leishmania* species causing human disease, except *L. donovani* from *L.infantum* [55]. Recently, an alternative technique, PCR-fluorescent fragment length analysis (PCR-FFL), has been developed by Tomás-Pérez et al, for use in *Leishmania* while its use has been reported previously for species identification in Trypanosoma [100,101]. In this study the fluorescently tagged primers used, were designed in the rRNA fragment ITS1 and 7SL region. The amplified fragments were digested and their sizes were determined by an automated DNA sequencer. PCR-FFL was found to be accurate and more sensitive than PCR-RFLP analysis [101].

Regarding the hsp70 PCR-RFLP approach, it is considered to be useful for the *L.(Viannia)* species discrimination while its sensitivity is poor for *L.(Leishmania)* species. Diagnostic RFLP patterns for the *L.guyanensis* species complex as well as for *L. lainsoni* and *L. shawi* are produced after restriction with the enzyme HaeIII [84,102]. However, this assay was not able to discriminate between *L. braziliensis* and *L. peruviana* as well as *L. naiffi*, requiring a second restriction enzyme for the differentiation [102] while also *L. guyanensis* and *L. panamensis* both belonging to the *L. guyanensis* complex share identical RFLP pattern [83]. The discrimination of the species mentioned above is of great significance due to the fact that even if *L. braziliensis* is considered to be the main causative agent of MCL [103] other *L.(Viannia)* species are also suspected of causing MCL. Additionally, a differential response to antimonial treatment has been documented [104–106]. This assay was suggested to be applicable on clinical samples [107,108].

Montalvo et al, extended the use of the hsp70 PCR-RFLP for identification of Old World and additional New World species and improved resolution within New World species complexes [108]. Recently, they developed an adequate and flexible toolbox which consists of one improved and three new PCR approaches based on hsp70 target amplification and subsequent RFLP, able to diagnose and identify the most medically relevant New and Old World *Leishmania* species. The new PCR variants were highly sensitive and specific and they presented improved amplification efficiency in clinical samples compared to hsp70 PCR described previously by Garcia et al [84]. The choice of the most suitable PCR among the four described, depends on factors like the origin of infection, the sympatry of species, the imported versus endemic pathology, the clinical presentation and the clinical sample [109].

Fernandes et al first developed a PCR approach based on mini-exon gene [36] which was later adapted by Mauricio et al. In this study the mini-exon PCR-RFLP was compared with ITS1 PCR-RFLP. Both targets were shown to be able to identify the strains studied but mini-exon was found to be more polymorphic than ITS1 whereas neither ITS1 nor mini -exon produced as many robust groups as gp63 based restriction analyses published before [91,95]. Marfurt et al also developed a mini-exon PCR-RFLP assay [39]. The pair of primers deriving from the conserved region was able to amplify DNA from Old and New World *Leishmania* species while

the diversity detected in the non transcribed spacers represented an informative phylogenetic marker. The digestion of the PCR products with one or two different restriction enzymes resulted in species-specific patterns allowing the species differentiation. Thus, they designed a mini-exon PCR-RFLP genotyping scheme, using different restriction enzymes. However, a single EaeI digest was informative enough for the speciation needed in clinical setting [39]. Furthermore, the repetitive character of this template made it highly sensitive even when applied to clinical samples [38]. On the other hand, when Bensoussan et al compared three PCR assays (kDNA,ITS1 and mini-exon used as targets) found that mini-exon presented the lowest sensitivity (53.8%) and suggested that this discrepancy may be attributed to the examination of stored clinical samples collected on filter papers instead of fresh samples, the extraction or the purification technique [110]. Rocha et al also adapted the PCR approach of Fernades et al and compared four PCR assays (kDNA and mini-exon used as targets) for the evaluation of New World *Leishmania* strains typing. Species belonging to the subgenus *Leishmania* were not amplified with the mini-exon target and the author suggested that this difference probably resulted from intraspecific variation [111]. Recently, in another study, ITS1 and mini-exon targets were compared with 18SrRNA in terms of sensitivity and discriminatory power in clinical samples, under routine laboratory settings. A new pair of primers for mini-exon target was designed due to the inability of the previous published primers to amplify the target in all clinical samples while also the protocol was slightly modified in order to achieve better diagnostic sensitivity. However, ITS1 was found to be more sensitive and practical than mini-exon. In contrast, mini-exon was again more polymorphic and revealed a great discriminatory power in *L.(Viannia)* subgenus [32].

The *L.donovani* complex is the causative agent of visceral leishmaniasis, the most severe form of the disease. The discrimination between the representatives of *L.donovani* complex, *L.infantum* and *L.donovani*, is important as they are morphologically indistinguishable while also they are associated with different epidemiology, ecology and pathology as *L.donovani* is anthroponotic and *L.infantum* is anthropozoonotic. Moreover, there are not discriminative markers to identify certain strains which status is questioned. Thus, the development of molecular tools capable of identifying diagnostic markers and allowing a better understanding of phylogenetic relationships is of great importance. In a study a PCR assay based on cysteine proteinase B (cpb) was developed which was able to differentiate between the two species. The cathepsin-1 proteases CPB which belong to the papain-like superfamily, clan CA and family C1, play an important role in the host protein destruction and evasion of the host immune response [88,112]. CPB enzymes are encoded by a tandem array located in a single locus. Mundodi et al, have compared a *L. donovani* strain and a *L.chagasi*(syn *L.infantum*) strain and revealed at least five tandemly arranged genes [113]. Hide and Banuls, used the last repeats of the cluster (cpbE for *L.infantum* and cpbF for *L.donovani*) and designed a PCR assay able to differentiate the two species by their fragment length as *L.donovani* strains were characterized by a 741-bp product and *L.infantum* strains by a 702-bp product. This PCR assay did not generate amplification for other *Leishmania* species nor trypanosomatids. Although sensitive and specific in cultured parasites, the assay is not sensitive enough for diagnosis on clinical samples [88]. The fact that the species discrimination is based on 39 bp difference in PCR product may cause problems in species identification when using normal agarose gel electro-

phoresis and where both species are not available for comparison. Thus, another cpb PCR assay was developed with subsequent digestion with DraIII which cuts the 741-bp amplicon of *L.donovani* into 400 and 341 bp and a PCR using a species-specific primer pair capable of amplifying a 317 bp of *L. donovani* whereas it did not amplify *L.infantum* [89]. Two cpb PCR-RFLP and one fluorogenic PCR assay for the molecular typing of *L.donovani* complex have been also developed and it was reported that the assays described were valid and informative for *Leishmania* typing in clinical samples [90,114]. Furthermore, a multilocus approach, using new and previously reported targets including cpb genes, was applied to neotropical isolates (*L.braziliensis, L.peruviana, L.guyanensis, L.lainsoni and L. amazonensis*) and was shown to be a highly robust method of distinguishing different strains [87].

Real-time PCR is considered to be a useful, sensitive, accurate and rapid tool for detection, quantification and even genetic characterization of *Leishmania* parasites. A LightCycler RT-PCR assay based on fluorescence melting curve analysis of PCR products generated from the minicircles of kDNA was developed. This assay was able to detect and differentiate four Old World *Leishmania* species (*L. major* was differentiated from *L. donovani* and from *L.tropica* and *L.infantum*) [45]. In another study, a qPCR based on glucosephosphate isomerase (GPI) gene was able to discriminate between subgenus *Viannia* and the complexes *L.mexicana, L.donovani/ infantum* and *L.major* [115]. A qPCR based on glucose-6-phosphate dehydrogenase (g6pd) using either SYBR-Green or TaqMan probes has also been described. This assay was able to differentiate *L.braziliensis* from other *L. (Viannia)* species and from those of *L.(Leishmania)* [116]. Weirather et al used a set of primers and probes for serial qPCR assays based on kDNA which was able to detect and differentiate *Leishmania* species in clinical samples due to different melt temperature of the amplicon or by observing the presence or absence of some amplicons [117]. Recently, tryparedoxine peroxidase gene was used as amplification target in a qPCR assay able to identify Old-World *Leishmania* species causing CL [54]. An alternative 18S rDNA based qPCR using fluorescence resonance energy transfer probes (FRET) was able to discriminate the *L.donovani* complex, the *L.brasiliensis* complex, and species other than these based on the distinct melting temperature obtained [46]. Finally, a new qPCR assay based on FRET technology and melting curve analysis was designed based on mannose phosphate isomerase (MPI) and 6-phosphogluconate dehydrogenase (6PGD) genes which found to be highly sensitive and discriminative for the five species of *Leishmania* being evaluated (*L.braziliensis, L.panamensis, L.guyanensis, L.peruviana* and *L.lainsoni*) [118].

MLEE, the technique which is regarded as the 'gold standard' for the identification of *Leishmania* parasites to species and subspecific levels and for genetic diversity studies, has been widely used since its introduction [119]. MLEE detects different alleles of housekeeping genes indirectly by scoring the electrophoretic mobility of the enzymes they encode. The nucleotide differences in the genes encoding the enzymes are reflected by their mobility differences. Thus, the parasites are identified by their enzymatic profile and are grouped in taxonomic units termed zymodemes, each one of whom consists of all the strains showing exactly the same profiles for all the enzymatic systems under study. Distinct combinations of isoenzyme mobilities for up to 15 enzymes have been assigned zymodeme numbers (MON-1–MON-274) [120].

However, this molecular method presents several disadvantages including the need for mass culture of *Leishmania* parasites and large amount of protein, it is timeconsuming, labour-

intensive, costly and technically demanding. It is also worth mentioning that the MLEE methods used in Europe and in South America are based on different enzyme panels and cannot be compared directly [74,93,119]. As far as it concerns its discriminatory power, it is considered to be poor due to its inability to detect nucleotide substitutions that do not change the amino acid composition and changes in the amino acid composition that does not modify the electrophoretic mobility. The discriminatory power of MLEE for classifications below species level is limited. For instance, the *L.infantum* zymodeme MON-1, the causative agent of the majority of visceral leishmaniasis cases around the Mediterranean basin and South America, has been shown to be genetically heterogeneous and polyphyletic with molecular markers presenting higher resolution level [121,122]. Moreover, other molecular studies do not always agree with the classification of *Leishmania* parasites by MLEE. For instance, the differentiation between the representatives of *L.donovani* complex, *L.donovani* and *L.infantum*, is based on only one enzymatic system (glutamate–oxaloacetate transaminase-GOT) making the species distinction poor. In fact, the zymodeme MON-30 which was regarded as *L.infantum*, has recently shown to be *L.donovani* [123,124]. Furthermore, the existence of *L.archibaldi* as a distinct species belonging to *L.donovani* complex was supported by MLEE but it was not in agreement with the results of many different molecular markers [125] while also *L.killicki* was not confirmed to be a separate species [94,126] and *L.donovani* zymodeme MON-37 was assigned to strains of different genetic background [74,120,127]. However, the codominant character of this molecular tool is advantageous as it is able to identify heterozygous profiles and thus potential hybrids while also if the proteins are highly polymeric, the distinction can be made between a heterozygous profile and a mixed infection [120].

Randomly Amplified Polymorphic DNA (RAPD), a simple process, distinct from the PCR, based in the amplification of genomic DNA with short oligonucleotides of arbitrary nucleotide sequence used as primers, has been also applied for *Leishmania spp*. The primers are designed and used for the detection of polymorphisms without relying on prior knowledge of the DNA sequence to be amplified [128]. From the advent of RAPD technique [128,129] numerous studies, only a few of them can be cited here, have been published reporting the use of RAPD as a molecular tool for *Leishmania* species identification and strain characterization. RAPD has been used for the investigation of the genomic diversity of *L.braziliensis* strains [130,131], *L.major* isolates [132], *L.donovani* complex [124,133,134] and *L.infantum* [77,121,135]. Regarding the use of RAPD in species identification, it has been applied for the differentiation between the species *L.braziliensis, L.mexicana, L.infantum, L.tropica, L.chagasi, L.amazonensis and L.major* [136], the identification and differentiation of Old World species at complex level [137] and recently for the characterization of clinical isolates responsible for kala-azar in India [138]. The main disadvantages of this technique are the need for parasite culture due to the use of non *Leishmania* specific primers and the poor reproducibility of the assay. Moreover, the bands of equal electrophoretic mobility may not be homologous and it is impossible to distinguish homozygous from heterozygous genotypes at specific loci because it is difficult to recognize allelic variants of randomly amplified polymorphic DNA markers in the absence of crossing data [74,120]

PCR hybridization is one of the first molecular methods for species identification and genotyping. DNA probes have been designed for *Leishmania* species identification. The most

common target used for *Leishmania spp* identification is kDNA. DNA probes targeting kDNA have been applied for *L.major* [139], *L.infantum* [140], *L.aethiopica* [141], *L.mexicana* and *L.braziliensis* [142], and *L.mexicana*, *L.donovani* and *L.braziliensis* complexes [143]. Other specific probes developed include a cDNA probe, designed from a repetitive degenerate sequence isolated from *L.donovani*, which specifically hybridized only with isolates of the *L.donovani* complex [144] and two probes, the pDK10 and the pDK20, which were able to differentiate between the Old World *Leishmania* species belonging to *L.donovani* complex and between all Old World *Leishmania* species respectively [145,146]. DNA probes generated from mini-exon genes have also been developed [147]. Other probes developed so far include a *L.braziliensis* specific probe [148] and *L.guyanensis* specific one [149].

MLEE has been recently modified in a direct sequencing allele detection method at each locus, called MLST. Partial sequences of approximately 700 bp in size, belonging to a defined set of housekeeping genes, are directly compared; the alleles are scored as identical or not and the same allele combinations are referred as sequence types. Alternatively, data analysis by sequencing of the alleles may be implemented. This technique was first used for bacterial pathogens whereas in *Leishmania*, steps have been taken to develop a MLST system [150]. The *L. donovani* complex has been studied by 2 sets of 5 loci for genes coding for enzymes used in MLEE [151,152]. These 10 targets in combination should be a complete MLST system for application in *L. donovani* complex. These studies showed that results from MLST are in agreement with results from MLEE whereas some discrepancies were found and MLST presented higher resolution level such as a silent Single Nucleotide Polymorphism (SNP) in gpi that distinguishes between strains of *L.infantum* [151]. Moreover, SNPs resulting in amino acid changes were also found in genes coding for enzymes giving indistinguishable electrophoretic profiles such as in nh2, which has the same protein band size for all *L.donovani* complex strains. These authors reported that MLST could be applied directly to clinical samples or to small-volume cultures. Furthermore, it can be used to detect recombination indirectly and for population genetics studies [151]. Tsukayama et al investigated the intraspecific and interspecific variation in the coding sequences of four enzymes (gpi, mdh, mpi and 6pgd), used in the MLEE typing method, in order to identify SNPs able to discriminate among closely related species. The assay was applied to clinical samples and successfully identified the species of *Leishmania* responsible for the clinical disease [153]. However, the analysis did not include sufficient diversity of strains for each species [74]. Recently, in another study a combination of the previous published enzyme-coding genes (fh, g6pdh, icd, mpi and pgd) was used so as to differentiate the Chinese *Leishmania* isolates and to investigate their phylogenetic relationships [154]. MLST is likely to become the gold standard basis for taxonomy and identification of *Leishmania*.

MLMT is based on the amplification of microsatellites sequences, tandem repeats of a simple nucleotide motif, 1-6 nucleotides, which are distributed abundantly in the eukaryotic and prokaryotic genomes and may reveal important strain polymorphisms. These markers are very useful for studying genetic variation between closely related organisms. Length polymorphisms in microsatellites sequences result from gain and loss of single repeat units which can be detected after amplification with specific to their flanking regions primers. MLMT ap-

proaches developed so far for *Leishmania spp*, make use of sets of 14–20 unlinked microsatellite loci. Microsatellite loci with high discriminatory power and being suitable for characterizing closely related strains have been published for the *L.donovani* complex [155–158], *L.donovani* strains [127] *L.major* [159], *L.tropica* [126,160] and for species of the subgenus *L. (Viannia)* [161]. Moreover, as the genetic diversity of *L.infantum* strains has been the subject of intense interest, several studies used MLMT approaches for the evaluation of the genomic variation in *L.infantum* strains [122,135]. It is worth mentioning that when MLMT was compared with other molecular markers for strain typing of *L.infantum*, the results obtained with kDNA PCR-RFLP were comparable to MLMT. kDNA and MLMT presented the highest discriminatory power especially for the MON-1 strains discrimination and appeared to be the most adequate for strain fingerprinting. However, MLMT is advantageous over kDNA PCR-RFLP because of its better reproducibility and feasibility of inter-lab comparisons and the co-dominant character of the markers used, making MLMT suitable for population genetic studies [77]. MLMT is suitable for high-throughput analysis and the data obtained are reproducible and exchange-able between laboratories. Moreover, accurate, quality controlled microsatellite profiles can be stored in databases and compared between different laboratories. In contrast to MLEE, selection does not seem to act on polymorphisms in microsatellite length while also the codominant nature of these markers permits the detection of the allelic variants. MLMT can be used directly on biological samples without prior culture of the parasite. DNA extracted from specimens spotted on filter paper or glass slides or from old Giemsa stained microscope slides was successfully applied in MLMT approaches [155]. It is recommended to use a panel of 10–20 unlinked microsatellite markers in all studies for nearly every species because microsatellite sequences are prone to homoplasy. Additionally, polymorphic repeats are not conserved between different species of *Leishmania* [74,122,157].

4. Phylogenetic analysis

Phylogenetics is the study of evolutionary relationships among various groups of organisms (e.g., species or populations). Their relatedness is evaluated through morphological and molecular sequencing data. This analysis leads to a hypothesis about the evolutionary history of taxonomic groups, their phylogeny. Regarding evolution, it is considered to be a branching process. Populations are altered with time and may split into separate branches, hybridize or be eliminated. The order in which evolutionary events are assumed to have occurred is revealed and may be visualized in a phylogenetic tree.

As mentioned before, MLEE is still regarded as the reference technique for the identification of *Leishmania* species and subspecies. The data obtained from MLEE were analyzed by phenetic and cladistic techniques and led to the construction of the first phylogenetic tree of the genus *Leishmania* [162]. The latter, revealed the monophyletic origin of the genus *Leishmania* and its subdivision into two subgenera, the *L.(Leishmania)* and the *L.(Viannia)* subgenus. *L.(Leishmania)* included the Old World species and *L.mexicana* and complexes from the New World. *L.(Viannia)* subgenus was composed from the other New World species. As *Sauroleishmania* was considered to be a separate genus, the lizard species were not included in these studies. MLEE

has been applied to a great variety and amount of isolates in comparison to other molecular methods in the past 25 years, resulting in the current classification system [93,162]. Phylogenetics based on different molecular methods, has confirmed the previous suggested taxonomy of the genus *Leishmania* by MLEE. However, the existence of a larger number of species has been proposed.

PCR-based methods with subsequent RFLP or DNA sequence analysis of multicopy targets or multigene families, to the recently developed MLST and MLMT, have been applied for the identification of the *Leishmania* species being responsible for the disease and for epidemiological studies in different endemic regions, as well as for taxonomic, phylogenetic, and population genetic studies. These tools except from their enhanced sensitivity they are also able to distinguish *Leishmania* parasites at species and intraspecies level. As for phylogenetic studies, the sequence analysis of single-copy gene targets is preferred while also the recombination and the different mutation rates between lineages make the use of one gene less suitable for the phylogenic analysis of the Trypanosomatidae or its subgroups[163].

Several DNA targets have been used to reveal the phylogeny of the *Leishmania* genus including single-copy genes encoding the catalytic polypeptide of DNA polymerase a (polA), the largest subunit of RNA polymerase II (rpoIILS) [164] and 7SL RNA [86], the ITS [165,166], the N-acetylglucosamine-1-phosphate transferase (NAGT) gene [167], the mitochondrial cytochrome b gene (cytb) [168], and most recently, sequences of the hsp70 subfamily [83]. Sequence analysis of these targets led to the conclusion that the subgenera L. (*Leishmania*) and *L.* (*Viannia*) constitute distinct monophyletic clades and that species of the Old and New World are segregated within the *L.* (*Leishmania*) subgenus. *Sauroleishmania* species branched off between the *L.(Leishmania)* and *L.(Viannia)* subgenera as an independent taxon suggesting that lizard *Leishmania* might be derived from mammalian parasites [164] and that they should be regarded as a subgenus of *Leishmania* rather than an independent genus [99]. However, the fact that RNA and DNA polymerase genes presented higher evolution rate in the lizard *Leishmania* than in the mammalian *Leishmania* species set into question the exact taxonomic position of lizard parasites [164].

In another study, Cupolillo et al. based on various molecular criteria, suggested the division of the genus *Leishmania* into two sections, *Euleishmania* and *Paraleishmania*. *Euleishmania* consisted of the subgenera *L.(Leishmania)*, *L.(Sauroleishmania)*, and *L.(Viannia)*. *Paraleishmania* included *L. hertigi*, *L.deanei*, *L.colombiensis*, *L.equatorensis*, *L.herreri*, and strains of *Endotrypanum*. In the latter section, the parasites of hystricomorph rodents, *L.hertigi* and *L.deanei* and the remaining species that are mainly parasites of sloths were genetically different while strains of *Endotrypanum* formed a paraphyletic group [169].

More recently Fraga et al. analyzed the phylogeny of the genus *Leishmania* based on the hsp70 gene. In this study the isolates and strains used, were of different geographic origins. The resulting phylogeny supported that the monophyletic genus *Leishmania* consisted of three distinct subgenera, the *L.(Leishmania)*, *L.(Viannia)*, and *L.(Sauroleishmania).* The obtained phylogeny supported the following eight species: *L.donovani*, *L.major*, *L.tropica*, *L.mexicana*, *L.lainsoni*, *L.naiffi*, *L.guyanensis* and *L.braziliensis*. In some of these species, subspecies were recognized including *L.donovani infantum*, *L.guyanensis panamensis*, and *L.braziliensis peruvi-*

ana. The so far recognized species *L.aethiopica, L.garnhami, and L.amazonensis* did not form monophyletic clusters [83].

Several discrepancies were reported for the taxonomic status of species obtained by MLEE compared to DNA based sequences. It is worth mentioning that the existence of *L.chagasi* and *L. archibaldi* as distinct species, was not supported by any molecular analyses as *L.chagasi* cannot be distinguished from strains of *L.infantum* and should therefore be regarded as South American strains of *L.infantum* [170,171] whereas *L. archibaldi* is not a valid species [125,159]. Numerous molecular studies did not even support the monophyly of the two remaining species, *L.donovani* and *L.infantum* [83,164,168]. Therefore, it was proposed that *L.donovani* is the only species of the *L.donovani* complex [83] while *L.donovani infantum* was recognized as subspecies. Regarding other geographically defined genetic groups within *L.donovani*, it was suggested that they could be delimited. Furthermore, the status of *L.killicki* has been debated. MLEE analysis supported the classification of *L.killicki* as a separate species while other molecular methods proposed that it was identical to *L.tropica* [94,126,168]. At the same time, *L.tropica* clusters to a single branch with *L.aethiopica*, making it difficult to be distinguished by the most of the DNA-based phylogenies [83,86,168]. It was suggested that they may represent different subspecies of the species *L.tropica* which is however needed to be investigated with a larger number of strains. Another discrepancy concerns the existence of the *L.mexicana* complex species. The strains of *L.mexicana* and *L.amazonensis* species are overpresented in DNA based phylogenies while only one *L. garnhami* strain was analysed in the hsp70 trees. In the latter study, none of these species could be distinguished as a monophyletic clade and *L.mexicana* was the only recognized species [83]. These results are in agreement with previous published studies [164,172,173] whereas they are in contrast to others [86,165,168]. Thus, the *L.mexicana* complex should be investigated, including *L.venezuelensis* and *L.aristidesi* strains, in order to evaluate the species and subspecies constituting this complex. The same holds true for the *L.braziliensis* complex species. Several molecular phylogenies including hsp70, RAPD and MLEE, supported the distinction of *L.peruviana* from other strains of *L.braziliensis* [83,174] and it was recognized as a subspecies in the *L.braziliensis* complex. However, this classification was questioned by a study using monoclonal antibodies [175] and another one analyzing the microsatellite variation [161] which suggested that strains of *L.peruviana* were grouped together with strains of *L.braziliensis* from Peru and from the Acre State, a Brazilian region bordering Peru. The use of a sufficiently large number of strains from different areas of distribution is needed so as the taxonomic status of the repsesentatives of the *L.braziliensis* complex to be evaluated. Moreover, in different phylogenetic trees, strains of *L.guyanensis* and *L.panamensis* formed a monophyletic cluster which was then divided into two monophyletic subclusters. Thus, the existence of two subspecies within the species *L.guyanensis* was proposed. A possible explanation for these discrepancies reported in different studies regarding the taxonomic status of both *L.peruviana* and *L.panamensis*, is the application of different molecular markers and the analysis of different strains.

Several molecular methods including MLEE [93], PCR-RFLP of ITSrDNA [78] and PCR-RFLP and sequence analysis of the hsp70 gene [102], were also suggested the inclusion of *L.shawi* in

the *L. guyanensis* group. The same applies for *L.naiffi* whereas *L.lainsoni* was confirmed to be the most divergent species inside the *L.(Viannia)* subgenus [83,102].

Noyes et al. (2002) identified a parasite isolated from human cutaneous lesions. Both stains were analysed by MLEE and found to be identical to each other and distantly related to all other *Leishmania* species. The application of other molecular methods revealed a low support for both its position basal to all *Euleishmania* and its clustering with *L.enriettii*. Thus, it was suggested that this strain may cluster with *L.(Leishmania)* or *L.(Viannia)* or form a novel clade within the *Euleishmania* either with or without *L.enriettii* [176]. Recently *Leishmania* species isolated from clinical samples from immunocompetent and immunosuppressed patients in Thailand [177,178] and a focal CL outbreak in Ghana [179] were identified and named as *L.siamensis*. Furthermore, novel *Leishmania* species, genetically indistinguishable, were isolated from kangaroos, wallaroos, and wallabies, living in captivity in the Northern Territory of Australia, a region that was considered free of *Leishmania* parasites [180]. Additionally, autochthonous cases of CL in German and Swiss horses and in a Swiss cow could not be classified neither as Old World nor New World *Leishmania* species while they were found to be most closely related to *L.siamensis* [181,182]. Finally, two new *L.(Viannia)* species were described and named *L.lindenbergi* [183] and *L.utingensis*. The last one was represented by only one sample isolated from a *Lutzomyia tuberculata* sand fly. Although the sequence analysis of single-copy gene targets has shown to be informative, the use of several independent genes displaying different evolutionary histories is preferable [184]. Such genes have applied in MLST and provided new insights on taxonomy and evolutionary history of *Leishmania*. MLST is currently considered the most powerful phylogenetic approach, it has been shown to have high discriminatory power, reproducibility and transportability of results between laboratories. Thus far, there are 10 published MLST targets available for the *L.donovani* complex [151,152], most of which are also applicable to other Old World *Leishmania* [185] and 4 targets for the sub-genus *Leishmania (Viannia)* [153]. This should form a complete MLST system applicable to *Leishmania* parasites.

5. Conclusion

Molecular methods have revolutionised the diagnosis of leishmaniasis. A variety of target sequences has been used and evaluated in different clinical samples of parasite hosts. Regarding PCR based assays, they were found to be rapid, sensitive and discriminative at species or even strain level. However the diagnosis of leishmaniasis remains a scientific challenge. There is a gap between the scientific advances, diagnostics and management of *Leishmania* infections in the field which should be decreased and an urgent need for standardization, optimization and simplification of PCR based applications. In this context, there is a generalized effort to make these assays available mainly in endemic areas around the world which will have an impact in disease control.

The great scientific interest for species identification may be attributed to its significance in prompt diagnosis and prognosis of the disease, decision making regarding treatment and

control measures. Despite the abundance of the studies carried out and the molecular markers used so far, the species discrimination is still tough in several closely related species. Thus, molecular tools with high discriminatory power are currently under development, optimization and evaluation.

Many molecular tools have been used for the *Leishmania* phylogeny and the definition of its taxonomy. However, evaluation of the phylogenetic relationships of *Leishmania* species is not an easy task. Moreover, there is a need for simplification of the classification and a meaningful nomenclature of *Leishmania* genus particularly for the clinicians.

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