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Chapter 4

Molecular Tools for Understanding Eco-Epidemiology, Diversity and Pathogenesis of *Leishmania* Parasites

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Additional information is available at the end of the chapter

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1. Introduction

Protozoan parasites of the genus *Leishmania* are responsible of a large variety of clinical manifestations ranging from self-healing cutaneous forms (CL), through mucocutaneous lesions (MCL), to lethal if untreated visceral disease (VL). Nevertheless, there is no absolute correlation between a particular clinical form and a causative species [1]. For instance, parasites of the *L. donovani* complex are generally responsible for VL cases in the Old and New World but can also cause CL. Another example is the *L. tropica* species, which causes a CL form but its association to VL cases was occasionally reported [2]. Identification of *Leishmania* parasites is a central issue to patients' management and to control. Leishmaniases have a worldwide distribution but only absent in the poles regions, and in Australia where in spite of presence of the parasites in Kangourous no human cases were described. According to the WHO, 350 million people are at risk, with a prevalence of 12 million and more than 98 countries affected [3]. More than twenty species are responsible for leishmaniasis in humans. However, *Leishmania* species present very similar morphologies in their flagellated, promastigote forms and their intracellular, amastigote forms which renders necessary the use of molecular or biochemical assays for their identification and characterization (see for review [4]).

The current identification and classification of *Leishmania* is still based on isoenzyme typing, using multilocus enzyme electrophoresis (MLEE) (reviewed in [5]). This approach has been widely used for the identification of *Leishmania*, but several limitations were reported. Most importantly, differences in electrophoretic profiles were shown to be due to heterozygosity at a single nucleotide position [6–8]. Molecular studies showed also that zymodemes included distinct DNA genotypes [7,9]. Consequently, other molecular studies do not always agree with the classification of *Leishmania* by MLEE. The other limitations of MLEE are that it requires

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bulk cultures of parasites, it is time-consuming, and it can be performed only in specialized laboratories. Therefore, alternative DNA based tools and assays are increasingly developed and used for effective investigation and characterization of the parasites.

Indeed, the diversity of *Leishmania* species, their vectors and their reservoir hosts is a main feature of leishmaniasis, so consequently the transmission cycles are very much dependent on the environment and are very prone to changes. So not only parasite identification is needed to establish etiology of the disease and understand the pathogeny, but knowledge of parasite diversity and its population structure is also needed for a better understanding of eco-epidemiology and its changing trends. For this purpose, molecular tools have been developed to allow differentiation of *Leishmania* parasites at species and strain levels within environmental or patients' samples.

Molecular tools are based mainly on the amplification and subsequent restriction fragment length polymorphism (PCR-RFLP) of several targets including repeated gene families and coding and non-coding regions, or the sequence analysis of the products. Recently multilocus sequence typing (MLST) and multilocus microsatellite typing (MLMT) were also developed for *Leishmania* DNA typing. Each of these molecular markers or tools has its specific discriminatory power, advantages and drawbacks.

2. Parasite identification

2.1. Differentiation at the genus level

This is based on the amplification of the kinetoplast minicircle DNA (kDNA, about 10000 copies per cell) or the variable sequences of the small subunit ribosomal DNA genes (SSU rDNA, 40–200 copies per cell) [10–13].

kDNA and SSU rDNA primers were initially designed for Trypanosomatids including *Leishmania*, *Sauroleishmania*, *Crithidia* and Trypanosomes. They allow identification of *Leishmania* parasites only at the generic and/ or subgeneric level. Both targets have also been used for the development of real-time PCR assays in order to determine parasite burden in clinical samples [14,15].

2.2. Differentiation at the species level

The ability to distinguish between *Leishmania* species is crucial for a correct diagnosis of the disease as well as for making decisions regarding treatment and control measures. This is especially useful in areas where several *Leishmania* species co-exist.

Numerous PCR approaches have been published based on different coding and non-coding regions in the *Leishmania* genome. Different targets have been used, including the ribosomal internal transcribed spacer (ITS) [12,16,17], the mini-exon genes [18,19], gp63 genes [20,21], hsp70 genes [22–24] and cysteine proteinase B gene sequences (cpB) [25,26].

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2.2.1. Randomly amplified polymorphic DNA (RAPD) and anonymous markers

Randomly amplified polymorphic DNA (RAPD) technique is based on the PCR amplification of DNA fragments using only one short primer that was arbitrarily defined and thus could be applied to any organism without a prior knowledge on the genome [27]. Such primers correspond to decamers having 60-70 % GC content and no self-complementary ends, thus the number of primers that could be used is virtually unlimited. Only in few occasions, two primers were used for *Leishmania* DNA analysis [28]; primers longer than 10 mers like universal primers used in cloning technologies have been used in some instances [29–32]. A list of selected primers used for *Leishmania* characterization is reported on Table 1. The RAPD technique generates monomorphic or polymorphic banding patterns, analyzed upon electrophoresis on agarose gels (or other supports) like DNA fingerprints. Given the fact that in comparison studies absence of bands does not reflect absence of corresponding DNA fragment in the compared DNA [33,34], the analysis is based only on Jaccard (or equivalent) distance or Similarity index [33] that only takes into account the presence of bands. RAPD reaction is very sensitive to reaction conditions even when variations are minor. Relaxed conditions and particularly low annealing temperature underlie DNA amplification but concentration or batch (quality) of DNA, reaction component, additives, brand of DNA polymerase, thermocyclers impact size range, complexity and reproducibility of the amplification profiles [33,35]. Molecular mechanisms underlying *Leishmania* DNA amplification was proposed to be based mainly on DNA mutations occurring on potential priming sites, that seem to be in regions enriched for short repeated motives [34].

Potential of RAPD and a selection of 28 primers was assessed for the discrimination between members of the *Leishmania Viannia* sub-genus which have overlapping geographical distribution in Latin America and that were difficult to distinguish by the conventional PCR using the primers then available. The authors have identified primers able to distinguish the 4 species (*L. braziliensis*, *L. guyanensis*, *L. panamensis* and *L. peruviana*) in a pair wise way. They also addressed the reliability of the technique by developing a statistical measure of the variation range of Jaccard coefficient when comparing the parasites [33].

Primer	Nucleotide sequence 5'-3'	References	Primer	Nucleotide sequence 5'-3'	References
OPA-01	CAGGCCCTTC	[30,34,36,43,44]	OPR-16	CTCTGCGCGT	[38]
H4 (OPA-02)	TGCCGAGCTG	[31,41,46]	OPR-20	ACGGCAAGGA	[38]
C4 (OPA-04)	AATCGGGCTG	[41]	OPU-15	ACGGGCCAGT	[38]
A5, P8, (OPA-05)	AGGGGTCTTG	[31,38,41,48]	OPU-16	CTGCGCTGGA	[38]
A4, (OPA-07)	GAAACGGGTG	[30,31,36,41,43]	OPU-02	CTGAGGTCTC	[43]
OPA-08	GTGACGTAGG	[28,30,31,33]	ILO 509	TGGTCAGTGA	[42]
OPA-09	GGGTAACGCC	[31]	ILO 526	GCCGTCCGA	[42]
OPA-10	GTGATCGCAG	[28,30,31,36,38,43, 48]	ILO 872	CCCGCCATCT	[42]
A12 (OPA-12)	TCGGCGATAG	[41]	ILO875	GTCCGTGAGC	[41,42]

Primer	Nucleotide sequence 5'-3'	References	Primer	Nucleotide sequence 5'-3'	References
A15 (OPA-15)	TTCCGAACCC	[41]	ILO 876	GGGACGTCTC	[42]
D10 (OPA-20)	GTTGCGATCC	[41]	ILO 878	GTCGCGGAG	[42]
D8 (OPA-16)	AGCCAGCGAA	[41]	A5, C5	CTCACGTAGG	[39,41]
OPB-01	GTTTCGCTCC	[30]	C6	CTGATCGCAG	[41]
C (OPB-04)	GGACTGGAGT	[28,33,43,44]	L2	CGGACGTCGC	[41]
B5 (OPB-05)	TGCGCCCTTC	[41]	H1	CGCGCCCGCT	[39,41]
B6 (OPB-06)	TGCTCTGCCC	[41]	L15996	CTCCACCATTAGCACCC AAAGC	[29,32]
OPB-07	GGTGACGCAG	[30,41]	λg11R	TTGACACCAGACCAACT GGTAAT	[29,32]
OPB-08	GTCCACACGG	[38,41]	M13a, M13	GTAAAACGACGGCCAG T	[30,33]
OPB-09	TGGGGGACTC	[30,33]	M13-40 F/ M13 (–40) a	GTTTTCCCAGTCACGAC	[29,30,32]
OPB-10	CTGCTGGGAC	[43,44]	M13/pUC	CGCCAGGGTTTTCCCAG TCACGA	[31]
OPB-12	CCTTGACGCA	[30,41]	P53-1	ACGACAGGGCTGGTTG CCCA	[32]
OPB-13	TTCCCCCGCT	[33,41]	PLiD2-9	CAAAAGTCCCCACCAA TCCC	[42]
OPB-15	GGAGGGTGTT	[30,43]	QG1	CCATTAGCACCCAAAG CAGACCTCACCCTGTGG AGC	[29,32]
A (OPB-18)	CCACAGCAGT	[28,30,33]	TA150	ATGCGATGAGTGGTTG AG	[41,42]
OPF-01	ACGGATCCTG	[38]	TA610	TCAACCGATTACAAACC A	[42]
OPF-10	GGAAGCTTGG	[43,44]	UMS	GGGGTTGGTGTA	[31,46]
OPF-13	GGCTGCAGAA	[38,43]	37	TGGATCCGGAATTTCGG CTTCACTAC	[42]
OPN-13	AGCGTCACTC	[38]	198	GCAGGACTGC	[41]
OPN-20	GGTGCTCCGT	[38]	233	CTATGCGCGC	[35]
OPR-13	GGACGACAAG	[38]	283	CGGCCACCGT	[35,48]
OPR-14	CAGGATTCCC	[38]	3301	TCGTAGCCAA	[30,33]
OPR-15	GGACAACGAG	[38,43,44]			

Name and sequence of the primers are reported on the table as described in the references. However, for the purpose of this work all the sequences were compared to lists provided by Operon Technologies (OP); primers thus identified are reported within brackets. Primers presenting discrepancies were not reported.

Table 1. Selection of primers used in RAPD analyses of *Leishmania* parasites generating polymorphic patterns.

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We have used the RAPD technique to identify and discriminate Old World species using 57 strains from different hosts, countries and reservoirs. Six random primers were tested from which 3 allowed to distinguish *L. aethiopica, L. arabica, L. donovani, L. major, L. tropica and L. turanica* species. We have analyzed the RAPD profiles considering criteria of consistent presence of amplified bands at the same electrophoretic presence for strains/ isolates of the same species, and the discrimination between parasites belonging to different species. This constitutes a simpler way to results interpretation that emphasizes on presence of consistently amplified and discriminative bands within a profile to overcome lack of reliability of RAPD [36]. RAPD also allowed differentiating Old World *Leishmania* species from the often co-sympatric *Sauroleishmania* parasites [30,36].

Random amplification of polymorphic DNA has been also used alone or with other techniques to confirm taxonomic status of parasites, for instance putative natural hybrids such as *L. braziliensis*/*L. panamensis* hybrids isolated in Nicaragua [33] or *L. braziliensis* and *L. panamensis*/ *guyanensis* in Ecuador [37]. In another example, genetic analysis of *Leishmania* parasites in Ecuador with MLEE and RAPD questioned the separation of *L. panamensis* and *L. guyanensis* as distinct taxa as these tools failed to generate clearly distinct clusters of parasites [38].

The RAPD technique was also used to investigate genetic diversity within *Leishmania* species or complexes in diverse settings. Causal agents of visceral leishmaniasis belong to the *L. donovani* complex, which includes the species *L. donovani*, *L. infantum*, *L. chagasi* and *L. archibaldi* [39]; however, taxonomy within this complex is controversial considering for instance *L. infantum* as forming its own complex [40]. The RAPD technique has been used to investigate intraspecific diversity of the *L. donovani* complex, using an initial set of 43 random primers [41]. Like in other studies [36,42], some primers differentiated the *L. donovani* complex from the other Old World taxa. Seven distinguished within the complex, differentiating in the tested panel of DNAs, Mediterranean *L. infantum* from the other parasites of the complex. Strikingly, none of the primers distinguished *L. donovani*, *L. infantum* and *L. archibaldi* taxa. Geographical clustering was observed with 2 strong Indian and East African *L. donovani* groups and a third Mediterranean *L. infantum* group in support to a previous study using RAPD in addition to other DNA tools [39]. Distribution of other *L. infantum* in the dendrogram also supports the paraphyly of *L. infantum* [41].

L. infantum zymodeme MON-1 has a worldwide distribution and is responsible mainly for a form of VL. RAPD analysis contributed to describe heterogeneity within this zymodeme and to demonstrate its polyphyletic nature [43]. The RAPD technique also highlighted geographical structures of *L. infantum* in diverse settings. In [42] they have shown that 17 (out of 18) primers tested on 33 strains isolated from diverse hosts in various Spanish regions generated highly polymorphic RAPD patterns that grouped the parasites into two main clusters that included parasites from central–western region in one side and from eastern Spain in the other. This study in addition illustrated intra-zymodemic diversity and lack of correlation with the MLEE analysis conducted on these strains, the clinical or host origin of the parasites. In another example, 53 *L. infantum* isolates from VL cases and dogs originating from different endemic regions in Brazil were analyzed with 5 RAPD primers (also used in [43]), MLMT and SSR-PCR. RAPD analysis was shown to be the most appropriate to illustrate genetic diversity of the

parasites. Interestingly, in spite of the homogenous genetic background the polymorphisms observed demonstrated correlation with geographical origin [44].

In Corte Pedra, North Eastern Brazil, *L. braziliensis* is causing different American Tegumentary Leishmaniasis (ATL) forms. Forty-five *L. braziliensis* strains isolated from patients having different ATL forms were shown to generate with 3 primers and 4 protocols, RAPD patterns having overall more than 80% fingerprint identity classifying the parasites into 5 clades. Significant distribution frequency of the different clinical forms along the clades was observed. The authors thus concluded on the suitability of the RAPD analysis of parasite strains' variability in Corte Pedra and that in such a spatially limited population geographical isolation precludes geographic sequestration as the mechanism for the observed genetic structures. In addition they assumed that infection with some *L. braziliensis* genotypes could be accompanied with different pathogenic mechanisms [28]. Other studies investigating *L. braziliensis* diversity in Brazil with other primers also highlighted contrasted diversity extent of parasites isolated from cutaneous leishmaniasis according to the transmission areas; parasites from Mato Grosso [32] or from Para [29] states were more diverse than the ones in Minas Gerais. These authors proposed that eco-epidemiology of the parasites in relation to environmental and geographical differences could explain in part such diversity patterns. Genetic diversity using intergenic region typing (ITSrDNA PCR-RFLP) and MLEE of *L. braziliensis* from diverse hosts and geographical origins in Brazil also illustrated occurrence of geographical clusters of parasites exerting different levels of variability; association of *L. braziliensis* to specific transmission cycles likely reflecting adaptation of different parasite clones to the vector (and diversity of) species involved in the transmission has been inferred [45].

The RAPD technique has also been used to investigate epidemiology of leishmaniases, characterizing clinical or field isolates in diverse settings. For instance, in India the increasing reports on drug resistance of the VL patients and the implication of *L. tropica* as a causal agent of VL, also hypothesized to be a potential reason for drug unresponsiveness [2], has prompted investigations of the causal agents of VL using various DNA tools. A first study for example characterized by MLEE and 8 RAPD primers 15 clinical isolates collected over 20 years from the eastern part of India from confirmed VL patients; this sample study comprised 1 PKDL and 6 antimony unresponsive cases. All parasites proved to be *L. donovani* [31]. Another study investigated with the same primers 9 other parasites isolated over the period 2006-2010 from hospital clinics in Kolkata from confirmed VL and a PKDL cases; one parasite was similar to *L. tropica* while the others were very close to *L. donovani* [46]. The association of *L. tropica* with the disease was further confirmed in another study using ITS, ITS1 and HP70 based assays [47]. In Iran, where cutaneous leishmaniasis is highly endemic, MLEE and kDNA were used to identify species of 565 parasites obtained from confirmed CL patients from the different provinces of the country during the 2002–2008 period [48]; this study associated *L. major* mainly to rural transmission and *L. tropica* to urban settings. RAPD using 3 primers allowed describing extensive genetic heterogeneity of a random selection of 65 *L. major* strains across the different transmission area and within the same foci.

In addition, RAPD technique constitutes a powerful alternative to the identification of PCR targets and markers. RAPD markers have been exploited for the design of species or complex

specific PCR assays like for instance a PCR that only amplifies DNA of parasites of the *L. donovani* complex [34] or another that amplifies exclusively *L. braziliensis* [49]. Such markers proved to be highly informative as probes or as PCR targets [34].

Randomly amplified polymorphic DNA products were used to develop markers that were targeted to develop typing strategies. For example, RAPD products that were amplified consistently across tested DNAs with a combination of 2 primers have been selected and sequenced partially to design marker specific PCR primers. The resulting PCR products were then screened for single stranded conformation polymorphisms (SSCP) and subsequently confirmed by sequence analysis [50]. This sequence confirmed amplified region analysis (SCAR) approach was used to differentiate 29 *L. donovani* strains from Sudan, Kenya, India and China using 8 different markers. The study identified 19 unique multilocus genotypes and a correlation between genotypes and geographical origin; SCAR markers were considered as co-dominant for their ability to detect all possible allele combinations in a diploid organism and as a representative random sample of neutral genetic variation in natural populations thus constituting appropriate tools for population studies [50].

Alternatively, with the objective to identify markers and develop simple assays for the discrimination of viscerotropic parasites encountered in Africa, we have screened 5 Operon kits (100 primers) for reproducible profiles and a selection of 28 primers was then used to screen for DNA markers within a panel of viscerotropic parasites from different countries in Africa and India [51]. These primers organized the parasites according to their geographical origin in a similar way to other studies using RAPD or other types of tools [39,41]. Some of the differentially amplified RAPD bands obtained in our study were cloned and sequenced; their analysis with bioinformatics tools and comparison to their respective genomic sites in *L. infantum*, *L. donovani* and *L. major* genomes highlighted the markers' association with simple sequence repeats and microsatellites in non coding regions [51]. A selection of such markers in *L. archibaldi* was used to develop simple PCR assays differentiating viscerotropic parasites, some of which in a country–specific way [52].

Randomly amplified polymorphic DNA is highly suitable for analysis of cultured *Leishmania* promastigotes but of limited interest for analysis of patients or zoonotic samples due to sample contamination with host DNA. Its use could be however contemplated to characterize promastigotes at the isolation step given the technique does not require large amounts of DNA (20 ng or less). Another generally admitted drawback is the lack of reproducibility generated by complex reactions occurring under the relaxed reaction conditions, therefore inter-laboratory or inter-study comparison using the same primers appear difficult to achieve. Options to overcome this drawback were the prior selection of primers of interest, or the use of defined criteria for analysis as for example relying only on consistently observed bands within RAPD patterns to assign the parasites to taxonomic groups [36] or using statistical tools to assess significance of the range of distances evaluated [33]. Use of well–standardized protocols may also help overcoming such a drawback. Although simple and having potential for detecting variation where other techniques fail, other drawbacks of this technique could be that bands of equal electrophoretic mobility may not be homologous [34]; identification of allelic variants is also not possible in *Leishmania*. Yet, RAPD constitutes a powerful tool for the

identification of markers and the design of PCR based assays [34, 49–52]. Diverse RAPD *Leishmania* studies reached conclusions that were confirmed by other tools or alternative studies making the RAPD approach still valuable.

2.2.2. Gp63 PCR-RFLP and sequencing analyses

Gp63 genes encode for the major metalloprotease of *Leishmania,* which is the most abundant surface glycoprotein found in promastigote and amastigote forms of the parasite. GP63 protein is encoded by a cluster of tandemly repeated genes, and has been identified as a virulence factor in several *Leishmania* species. Several groups have studied the potential of gp63 as a species discriminatory tool in *Leishmania*. Amplification of gp63 genes coupled with restriction analysis (PCR-RFLP) was applied to a large number of isolates belonging to 4 species of the subgenus *Viannia*, namely *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *lainsoni* and allowed discrimination of all the species tested [20] (Table2).

Target	Primers sequences (5'–3')	Product size (bp)	Discrimination by		Refs
			PCR-RFLP	Seq.	
kDNA	(F) Pia1: ACGAGGTCAGCTCCACTCC	100	-	-	[11,13]
	(R) Pia2: CTGCAACGCCTGTGTCTACG		-	-	
	(F) Pia3: CGGCTTCGCACCATGCGGTG	260	-	-	
	(R) Pia4: ACATCCCTGCCCACATACGC		-	-	
	(F) K13A : GTGGGGGAGGGGCGTTCT	120			
	(R) K13B: ATTTTACACCAACCCCCAGTT				
	(F) RV1: CTTTTCTGGTCCCGCGGGTAGG	145			
	(R) RV2: CCACCTGGCCTATTTTACACCA				
SSU rDNA	(F) R221: GGTTCCTTTCCTGATTTACG (R) R332: GGCCGGTAAAGGCCGAATAG	603	+	+	[10]
Gp63	(F) TDM1: GTCTCCACCGCAGACCTCACGGA (R) TDM2: TGATGTAGCTGCCATTCACGAAG	1300	+	-	[20]
	(F) SG1: GTCTCCACCGAGGACCTCACCGA	1300	+	-	[21]
	(R) SG2: TGATGTAGCCGCCCTCCTCGAAG				
	(F) PDD1: TCGGTGAGGTCCTCGGTGGAGAC	1700	+	-	
	(R) PDD2: CTTCGAGGAGGGCGGCTACATCA				
	(F) C9F: GGCTCCCGACGTGAGTTA	1750	+	-	[58]
	(R) C1R: GGGCCCGGGCGACAGCAGCGATGACTG				

	(F) C10F: GGGAAGCTTACGTACAGCGTGCAGGTG (R) C1R: GGGCCCGGGCGACAGCAGCGATGACTG	1600, 2000 and 4500	+	-	
ITS	(F) LITSV: ACACTCAGGTCTGTAAAC (R) LITSR: CTGGATCATTTTCCGATG	1040 or 950–1100	+ +	+ +	[64,65,69]
ITS1	(F) IR1: GCTGTAGGTGAACCTGCAGCAGCTGGATCATT (R) IR2: GCGGGTAGTCCI'GCCAAACACTCAGGTCTG	1000–1200	+	-	[16]
	(F) LITSR: CTGGATCATTTTCCGATG (R) L5.8S: TGATACCACTTATCGCACTT	300–350	+	+	[12,13,61,65,82]
ITS2	(F) LGITSF2: GCATGCCATATTCTCAGTGTC (R) LGITSR2: GGCCAACGCGAAGTTGAATTC	372–450	-	+	[63]
	(F) L5.8SR: AAGTGCG-ATAAGTGGTA (R) LITSV: ACACTCAGGTCTGTAAAC	720	-	+	[65]
ITS1and part of ITS2	(F) LITS-MG: ATG GCC AAC GCG AAG TTG (R) LITSR: CTGGATCATTTTCCGATG	800	-	+	[69]
Hsp70	PCR-G : (F) HSP70sen: GACGGTGCCTGCCTACTTCAA (R) HSP70ant: CCGCCCATGCTCTGGTACATC	1422	+	-	[22,72]
	PCR-F : (F) F25: GGACGCCGGCACGATTKCT (R) R1310: CCTGGTTGTTGTTCAGCCACTC	1286	+	-	[73–75]
	PCR-N : (F) F25: GGACGCCGGCACGATTKCT (R) R617: CGAAGAAGTCCGATACGAGGGA	593	+	-	
	PCR-C : (F) F251: GACAACCGCCTCGTCACGTTC (R) R991: GTCGAACGTCACCTCGATCTGC	741	+	-	
	(F) HSP70sen: GACGGTGCCTGCCTACTTCAA (R) HSP70ant: CCGCCCATGCTCTGGTACATC	1422	-	+	[23]
	(F) HSP70-F335 CACGCTGTCGTCCGCGACG (R) HSP70-R429 AACAGGTCGCCGCACAGCTCC	113	-	+	
	(F) HSP70-2F CTGAACAAGAGCATCAACCC (R) HSP70-2R CTTGATCAGCGCCGTCATCAC	170	-	+	
	(F) HSP70-F893 GTTCGACCTGTCCGGCATCC (R) HSP70-R1005 GTGATCTGGTTGCGCTTGCC	130	-	+	
	PCR-F : (F) HSP70-F25: GGACGCCGGCACGATTKCT (R) HSP70-R1310: CCTGGTTGTTGTTCAGCCACTC	1286	-	+	[76]
	PCR-T : (F) HSP70-6F GTGCACGACGTGGTGCTGGTG (R) HSP70-R1310: CCTGGTTGTTGTTCAGCCACTC	766	-	+	
	PCR-N : (F) HSP70-F25: GGACGCCGGCACGATTKCT (R) HSP70-R617 CGAAGAAGTCCGATACGAGGGA	593	-	+	

	3'UTR : (F) 70-IR-D: CCAAGGTCGAGGAGGTCGACTA (R) 70-IR-M: ACGGGTAGGGGGAGGAAAGA	516–733	-	+	[77]
Mini-exon	(F) Fme: TATTGGTATGCGAAACTTCCG (R) Rme: GAAACTGATACTTATATAGCG	220–443	+	+	[19,13,78]
	(F) FME2: ACTTCCGGAACCTGTCTTCC (*Leishmania* subgenus) or ACTTCCGGGACCCGTCTTCC (*Viannia* subgenus) (R) ME2R: CAGAAACTGATACTTATATAGCGTTA	220–443	+	+	[82]
Cysteine protease B	intragenic region : (F) CPBFOR: CGAACTTCGAGCGCAACCT (R) CPBREV: CAGCCCAGGACCAAAGCAA	1079	+	-	[83,84]
	Intergenic region : (F) PIGS1A: CCTCATTGCTTTGGTCCTGG (R) PIGS2B: GGCGTGCCCACGTATATCGC	1600	+	-	
	cpbEF: (F) CGTGACGCCGGTGAAGAAT (R) CGTGCACTCGGCCGTCTT	702–741	-	-	[25,85]
	cpbEF: (F) CGTGACGCCGGTGAAGAAT (R) CGTGCACTCGGCCGTCTT	702–741	+	+	[26]
	Leishmania cpb: (F) LmcpbUNIF: ACGGTCTTAGCGTGCGAGTTGTG (R) LmcpbUNIR: CAAGGAGGTCCCCTCACGCG	1440	-	+	[85]
	L. major cpb (variant 1): (F) LmcpbUNIF: ACGGTCTTAGCGTGCGAGTTGTG (R) LmcpbR: TCGTGCAGCACATGTCGCTTG	1176	-	+	
	L. infantum cpb: (F) cpbEF For: CGTGACGCCGGTGAAGAAT (R) L. inf Rev: CGTTTCGTTGCTCGGGATCAT	325	-	-	
	L. tropica cpb: (F) LmcpbUNIF: ACGGTCTTAGCGTGCGAGTTGTG (R) Ltro Rev: ACAGGGCCGTCAGCCCGTGGC	600	-	-	
	L. infantum cpb: (F) infcpbE: GTCTTACCAGAGCGGAGTGCTACT (R) Inf2.1: ATAACCAGCCATTCGGTTTTG	278	-	-	[86]
	L. donovani cpb: (F) cpbF2.1: GCGGCGTGATGACCAGC (R) Do2.1: CAATAACCAGCCATTCGTTTTTA	309	-	-	
	(F) MATRAE2: GGCGATGGTGGAGCAGATGATCT		-	-	

(R) Ma4.1: CGGTTCTCGTAGCACACTTGTTG	99 (*L. major*)			
(R) Tr4.1: CTCCCCCGTTCGGAT	100 (*L. tropica*)			
(R) Ae2.1: AGTACGTGCACATCAGCACATGGG	154 (*L. aethiopica*)			
(F) V5F: GGTGATGTGCCCGAGTGCA (R) V10R: CGTGCACATCAGCACATGGG	564	-	-	
(F) CpbF: GTGCGTGCGGGTCGTGC (R) CpbR: AAAGCCCCGGACCAAAGCA	735	-	-	[87]

(F): Forward primer; (R): Reverse primer; Seq.: sequence analysis; Refs: references

Table 2. Overview of DNA targets, primers sequences, product sizes and the technique used to achieve discrimination of *Leishmania* taxa.

Gp63 PCR-RFLP tool was also used to characterize isolates representative of the *L. donovani* complex (*L. infantum*, *L. donovani*, *L. archibaldi* and *L. chagasi*), with special attention to Mediterranean *L. infantum* from different geographical origins, in addition to representative strains of Old World *Leishmania* (*L. major*, *L. tropica* and *L. aethiopica*) [21] (Table2). This allowed discrimination of the 4 species of the *L. donovani* complex, which were quite distinct from the outgroup. Within *L. infantum*, the parasites were found to be polymorphic showing a geographical structuring [21]. Sequences of the gp63 genes were explored in 33 strains of the *L. donovani* complex having different origins and zymodemes, in addition to reference strains of other Old World species [53]. Evolution of the gp63 multigene family was inferred to be under the influence of a mosaic or fragmental gene conversion mechanism. The sequences clustered according to the species, showing a concerted evolution of the different gene classes. Phylogenetic analyses confirmed the genetic diversity of the *L. donovani* complex, which showed that gp63 genes could provide the basis for rapid and reliable genotyping of strains in this complex [53].

Furthermore, still using gp63 coding sequences PCR-RFLP evaluated intra-specific polymorphism of *L. infantum* isolates in Tunisia [54]. In total, 22 *L. infantum* isolates responsible of visceral (14 isolates) and cutaneous (8 isolates) forms of leishmaniasis in Tunisia were analysed, in addition to reference isolates, representative of Old World complexes. The *Sal*I, *Hinc*II, *Bal*I and *Bsi*EI restriction enzymes were used in this intragenic gp63 PCR-RFLP analysis. Results showed profiles that allowed distinction of *L. infantum* from the other species belonging to the *L. donovani* complex (*L. donovani*, *L. archibaldi* and *L. chagasi*) but also from the Old World species *L. major*, *L. tropica* and *L. aethiopica*. Besides, polymorphic patterns were observed among *L. infantum* isolates that tend to be correlated to the clinical presentation of the disease; the phenetic analysis using a UPGMA clustering method (Phylip package) grouped all VL isolates together while most of the CL parasites clustered in a separate branch. Good bootstrapping values supported the clusters [54].

The gp63 PCR-RFLP method was applied to characterise parasites contained within the lesions of patients having cutaneous leishmaniasis, originating from areas in central Tunisia, known

to be free of CL. This analysis confirmed assignment of the parasites to the *L. infantum* species, thus demonstrating the occurrence/emergence of sporadic cutaneous leishmaniasis (SCL) due to *L. infantum* in central Tunisia [55].

The gp63 PCR associated to RFLP analysis was also used to characterise transmitted *Leishmania* in Sudan. Patients that presented with uncommon cutaneous leishmaniasis, including one case with a *L. tropica* like-lesion, were confirmed, using this tool, to be infected by *L. major* [56]. In another study, *Leishmania* parasites from Sudanese patients having cutaneous ulcers were analyzed by gp63 PCR-RFLP and shown to belong to the *L. donovani* species [56]. This work allowed concluding that, in addition to *L. major*, *L. donovani* species can also be a major cause of CL in Sudan [57].

Another PCR-RFLP analysis of the gp63 intergenic region was also developed and tested on the *L. donovani* complex [58]. The markers generated robust and congruent phylogenies, identifying 5 genetic clusters within *L. donovani* complex. Furthermore, clusters strongly correlated with isoenzyme typing and some with geographical origin, which may be important for epidemiological and clinical studies [58].

Although the gp63 PCR RFLP technique has been successfully used for *Leishmania* discrimination at the species and strains levels, it presents several disadvantages. The fragment patterns obtained are sometimes vey complex and can be difficult to analyze and compare between laboratories. Also, partial restriction needs to be carefully evaluated as a potential source of artefacts. This technique depends therefore, on careful standardization and is recommended for comparative studies involving few strains rather than for large-scale epidemiological studies. Given the size of the sequences amplified (1.3Kb for the intragenic PCR) and the number of (GC rich) copies, the PCR assay requires good quality DNA and additives like DMSO [21] and thus a careful establishment step.

2.2.3. ITS1 PCR–RFLP and ITS2 targets

Ribosomal RNA (rRNA) genes are highly repetitive and conserved sequences. The ITS1 region is the sequence in between the 18S rRNA and 5.8S rRNA genes. It has enough conservation to serve as a PCR target but sufficient polymorphisms to facilitate species typing. ITS1 PCR has been developed in combination with an RFLP analysis (Table2) with different restriction enzymes (*Alu*I, *BstU*I, *Eco*RI, *Fsp*I, *Hae*III, *Hha*I, *Rsa*I, *Sau3A*I, *Sph*I and *Taq*I) [16]; *Hae*III is the mostly used restriction enzyme used for species identification. Indeed, ITS1 PCR–RFLP using *Hae*III is the most widely used assay for direct detection and identification of *Leishmania* species in the Old World.

It has been applied for the distinction of sympatric species, especially in the Mediterranean region [59,60]. However, representatives of the *L. donovani* complex (*L. donovani* and *L. infantum*) and also of the *L. braziliensis* complex (*L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. peruviana*) cannot be distinguished by this approach, even using a great variety of restriction enzymes [12]. This limitation can however be bypassed by sequencing of the ITS1 PCR product thus allowing for a clear separation of these species and also assignment of different strains [61].

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Recently, real-time PCR product from the ITS1 region has been used in a high-resolution melt (HRM) analysis in order to identify and quantify Old World *Leishmania* species [62]. High resolution melt analysis is a molecular technique that uses a fluorescent intercalating dye to measure the rate of double stranded DNA dissociation consequent to an increase of temperature. The observed melting curve is characteristic of a particular DNA and depends on its sequence length, GC content, complementarity, and nearest neighbour thermodynamics. The dye is incorporated during the amplification; the DNA dissociation measures occur at the end of the PCR, which is performed in a dedicated thermocycler. The results are computerized and analyzed through a graphic output. When tested on 300 samples from human cases, reservoir hosts and sand flies, this approach distinguished all Old World *Leishmania* species causing human disease, except *L. donovani* from *L. infantum* [62].

The ITS2 region is located in between the 5.8S rRNA and LSU rRNA genes. It has been studied and found to be adequate for species identification. Indeed, generic PCR primers (LGITSF2/LGITSR2) were designed to amplify this fragment from *Leishmania* spp. associated with human infection, using reference isolates [63] (Table 2). Substantial differences in the ITS2 region amplified by these primers followed by sequencing analysis, allowed detection of and discrimination among *Leishmania* species from the Old and New World [63]. The ITS2 PCR followed by DNA sequence analysis approach was validated on clinical specimens, which allowed identification of a total of 8 *Leishmania* species (*L.* (*V.*) *braziliensis, L.* (*V.*) *guyanensis, L. (V.) panamensis, L. (L.) mexicana, L. aethiopica, L. major, L. tropica and the L. donovani* complex) among 159 patients corresponding to U.S. civilians that had travel and immigration history to leishmaniasis endemic countries [63].

The ITS1 and ITS2 region have also been used to assess intra-specific DNA polymorphisms among *L. donovani* isolates from different geographical origins [64,65] (Table 2). Single-stranded conformation polymorphism (SSCP), and sequencing of the ITS regions were applied to clinical samples of *L. donovani* from Sudan and one from Kenya, one from India and one from China. Intra-specific variation in SSCP banding patterns was clearly observed in the ITS1 region and gave five different SSCP profiles; 3 profiles were detected among Sudan isolates and 2 ITS1-SSCP profiles were observed among the samples from Kenya, India, and China [65]. This corroborates the results of a previous study in which 11 polymorphic ITS1-SSCP patterns were identified among 63 clinical samples of *L. donovani* from eastern Sudan [64]. On the other hand, no variation was observed in the ITS2 region among the 63 studied cases from Sudan [64] and the study that analyzed the ITS2 locus among 23 Sudanese samples, showed again the same ITS2 SSCP pattern, with the exception of 1 isolate that had a different one [65].

When the species *L. tropica* was studied using ITS1 amplification and SSCP analysis, 14 SSCP profiles within 29 strains from different Old World geographical areas were found [66,67]. The *L. major* species was also investigated for DNA polymorphisms using ITS1 and ITS2 PCR amplification followed by SSCP analysis and sequencing [68]. Results revealed in total five genotypic variants among *L. major* isolates from Iran [68].

Recently, authors from Iran used primers LITSR and LITSV to amplify whole ITS region and found a double banded electrophoretic pattern in *L. tropica* species, while a sharp single band was observed for *L. infantum* and *L. major* isolates [69]. In order to explain how this two-band

pattern occur in *L. tropica*, an *in silico* analysis of ITS sequences was conducted and showed the existence of two groups of sequences that differ by a 100bp gap, indicating existence of at least two alleles for ITS in ribosomal DNA. Thus, a specific reverse primer was developed (LITS-MG, Table 2) in order to amplify, with LITSR, sequences located just before the gap, which included ITS1 5.8S and a part of the ITS2 sequence. Amplification using LITS-MG/ LITSR primer set, followed by sequencing, allowed discriminating *L. tropica* from *L. infantum* or *L. major* [69].

Although PCR-RFLP of the ITS1 spacer is the most widely used assay for direct detection and identification of *Leishmania* species in the Old World, it has some limitations. Despite that all medically relevant *Leishmania* species can be distinguished by digesting the ITS1 PCR product with *Hae*III restriction enzyme, representatives of the *L. donovani* complex (*L. donovani* and *L. infantum*) or *L. braziliensis* complex (*L. braziliensis*, *L. guyanensis*, *L. panamensis* and *L. peruviana*) have almost identical RFLP patterns with a great variety of restriction enzymes and cannot be resolved further by this approach [12]. This problem can, however be solved partially by sequencing the ITS1 PCR product. Use of a highly resolutive agarose or SSCP analysis may be needed to resolve differences between some species or to investigate intra-specific polymorphism, respectively. ITS2 region has also served for species identification but the drawback of this approach is the need for DNA sequencing analysis. Sequencing or SSCP analysis may not be available in most laboratories in areas of endemicity.

2.2.4. hsp70 PCR–RFLP and sequencing

The 70kDa heat-shock proteins (HSP70) are encoded by genes that are highly conserved across prokaryotes and eukaryotes both in sequence and function. They have great importance as molecular chaperones in protein folding and transport [70]. Genes encoding cytoplasmic HSP70s were among the first kinetoplastid genes that were cloned and characterized because of their conserved nature [71]. HSP70 protein and its encoding gene have been widely used for phylogenetic and taxonomic studies of different parasites, including *Leishmania*.

The PCR-RFLP approach targeting hsp70 sequences has proven to be most useful for the differentiation between South American *Leishmania* species from the subgenus *Viannia* (Table2). Using the restriction enzyme *Hae*III to digest the amplified product, the produced RFLP patterns allowed discrimination between *L. guyanensis* species complex as well as for *L. lainsoni* and *L. shawi* [22,24]. However, *L. braziliensis* and *L. peruviana*, both belonging to the *L. braziliensis* complex, as well as *L. naiffi* showed an identical *Hae*III RFLP pattern. They can be distinguished by using other restriction endonucleases like *Mbo*I and *BstU*I [24]. The Hsp70 PCR-RFLP approach was extended for identification of Old World and additional New World species with an improved resolution within species complexes; in total 139 strains from 14 species were studied using *Hae*III, *Bcc*I, *Rsa*I, *Mlu*I, and *BsaH*I restriction enzymes [72]. Two subsequent digestions of the PCR products identified the species *L. infantum* and *L. donovani* (*Hae*III and *Mlu*I), *L. tropica* and *L. aethiopica* (*Hae*III and *BsaH*I), *L. braziliensis* and *L. peruviana* (*Hae*III and *Rsa*I), *L. guyanensis* and *L. panamensis* (*Hae*III and *Bcc*I); the first digestion using *Hae*III discriminates among the broad groups while the additional ones discriminate within these groups; the species *L. major*, *L. lainsoni* and *L. naiffi* had specific patterns with *Hae*III

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restriction enzyme, without need to use an additional digestion [72]. However, it was not possible to differentiate between the species *L. mexicana*, *L. amazonensis*, and *L. garnhami* [72].

In order to improve the sensitivity and specificity of the previously reported hsp70 PCR, alternative PCR primers and RFLPs were used [73] (Table2). Thus, three new PCR primer sets (PCR-F, PCR-N, and PCR-C) and their corresponding restriction scheme (RFLP-F, RFLP-N, and RFLP-C) were tested. The detection limit of the new PCRs was between 0.05 and 0.5 parasite genomes; they amplified clinical samples more efficiently, and were *Leishmania* specific. A specific discriminative power was found for each new RFLP analysis: in general species from the Old World (*L. major*, *L. tropica*, *L. aethiopica*, *L. donovani*, *L. infantum*) and from the New World (*L. infantum*, *L. lainsoni*, *L. peruviana*, *L. guyanensis*, *L. panamensis*) were well differentiated [73]. Discrimination of *L. guyanensis* and *L. panamensis* species, both belonging to the *L. guyanensis* complex is important for epidemiological purposes and has also consequences for the prognosis of the disease, since MCL, which is principally associated with *L. braziliensis*, can also be caused by other *L.* (*Viannia*) suspected species. Recently, an updated hsp70PCR RFLP protocol for RFLP-F and RFLP-N designed in [73] was published, with new restriction enzymes [74]. These new enzymes showed reduced cost and allowed better separation of some New World (sub)species [74].

Relevance of the hsp70 PCR-RFLP approach [72–74] is illustrated by a study that applied it on 89 clinical samples from a total of 73 Peruvian patients with either cutaneous or mucocutaneous leishmaniasis. The new PCRs were tested on tissue samples, lesion biopsies, aspirates, and scrapings. They showed an improved sensitivity both for genus detection and species typing and identified the species *L. braziliensis*, *L. peruviana* and *L. guyanensis* [75].

In addition to PCR-RFLP analysis, the hsp70 gene was also used in sequencing. Indeed, the 1380bp fragment of the coding region commonly used in RFLP analysis was sequenced in 43 isolates from different geographic origins for studying evolutionary relationships [23]. Fifty-two hsp70 sequences representing 17 species commonly causing leishmaniasis both in the New and Old World were analyzed. The authors found that the genus *Leishmania* formed a monophyletic group with three distinct subgenera *L. (Leishmania)*, *L. (Viannia)*, and *L. (Sauroleishmania)*. The obtained phylogeny supported the eight species *L. (L.) donovani*, *L. (L.) major*, *L. (L.) tropica*, *L. (L.) mexicana*, *L. (V.) lainsoni*, *L. (V.) naiffi*, *L. (V.) guyanensis* and *L. (V.) braziliensis*. In some of the species, subspecies *L. (L.) donovani infantum*, *L. (V.) guyanensis panamensis*, and *L. (V.) braziliensis peruviana* were recognized [23]. Recently, sequencing of the hsp70 gene was useful for *Leishmania* species determination within clinical samples, overcoming need for parasite isolation [76]. The results obtained were in great agreement with those from multilocus enzyme electrophoresis [76].

The 3′-untranslated region (UTR) of hsp70-type I gene constitutes an alternative target for sequence analysis [77]. These authors who used it to analyse 24 strains representing 11 *Leishmania* species, found a remarkable degree of sequence conservation in this region, even between species of the subgenera *Leishmania* and *Viannia*. In addition, the presence of many microsatellites was a common feature of the 3′-UTR of HSP70-I genes in the *Leishmania* genus. Global sequence alignments and resulting dendrograms demonstrated usefulness of this particular region of hsp70 genes for species (or species complex) typing, improving the

discrimination capacity of phylogenetic trees based on hsp70 coding sequences in case of some species (*L. donovani*/*L. infantum*; *L. tropica* and *L. aethiopica*; *L. braziliensis*/*L. peruviana*; *L. guyanensis*/*L. panamensis*) [77].

Using hsp70 gene in PCR followed by RFLP or sequence analysis presents many advantages. It is easily comparable across all *Leishmania* species worldwide and discriminates all relevant species in both subgenera *L. (Leishmania)* and *L. (Viannia)*. In addition, the approach has been optimized for direct amplification from clinical samples. However, systematic sequencing of the hsp70 gene for *Leishmania* identification purposes represents the major disadvantage of this approach, since this technique needs high-resource settings. For this, it was stated, "this method is especially suited for use in non-endemic infectious disease clinics dealing with relatively few cases on an annual basis, for which no fast high throughput diagnostic tests are needed" [76].

2.2.5. Mini-exon PCR-RFLP

The mini-exon genes are involved in the trans-splicing process of nuclear mRNA in kinetoplastid protozoa and are present as 100 to 200 tandemly repeated copies per nuclear genome. Mini-exon genes contain a highly conserved exon of 39 bp with a moderately variable transcribed intron region (55 to 101 bp) and a highly variable non-transcribed spacer sequence (51 to 341 bp). These genes were extensively used as a PCR target to identify and discriminate Old and New World *Leishmania* species [19,78]. This PCR assay amplified all the miniexon sequences in a single reaction (Table2). In addition, size variability of the amplification products allowed preliminary discrimination between the major complexes (Old and New World *Leishmania*, and New World *Viannia* complexes). After enzymatic restriction of the PCR product with *Hae*III or *Eae*I, a characteristic RFLP pattern is produced that depends on size variations in the polymorphic spacer regions as well as mutations in the recognition sites of the restriction enzymes. *Eae*I profiles were shown to be more informative than *Hae*III and allowed to distinguish between the most important Old World species, *L. major*, *L. tropica*, *L. aethiopica*, *L. infantum* and *L. donovani* [19,78]. However, with *Hae*III, species belonging to the *L. braziliensis* complex (*L. braziliensis* and *L. peruviana*) and to the *L. guyanensis* complex (*L. guyanensis* and *L. panamensis*) could be discriminated [19].

This genotyping method was successfully applied to naturally infected clinical samples for the differentiation of New and Old World *Leishmania* species and showed a high sensitivity and a robust and reliable species differentiation power [79]. Several other research groups have applied mini-exon PCR-RFLP method for identification and characterisation of *Leishmania* species, using various types of samples from different countries. In [80], they have analyzed microcapillary cultivated isolates from cutaneous and visceral cases in Turkey and identified the species *L. infantum* and *L. tropica* in CL cases, and *L. infantum* in VL ones. In Nepal, bone marrow aspirates from VL patients were analyzed by mini-exon PCR-RFLP and the parasites have been shown similar to the standard Indian strain of *L. donovani* and different from the Kenyan strain [81].

Recently, mini-exon PCR-RFLP was compared to the ITS1 PCR RFLP approach on a set of reference strains [82]. The ITS1 PCR proved to be slightly more sensitive and more practical than the mini-exon. Analysis using the ITS1 digested with *Hae*III allowed to distinguish most species but an additional digestion with *Cfo*I may be helpful in case of *L. mexicana*. However, using the mini-exon, sequencing was found to be the most practical approach as the mini-exon sequences add information since they are more polymorphic than the ITS1 sequences [82]. Therefore, the mini-exon genes were used for typing the species that belong to the *L. Viannia* subgenus, also known as *L. braziliensis* complex, which cannot be distinguished with the ITS1 [82].

2.2.6. Cysteine protease B (cpb) based PCR and PCR RFLP

Cpb genes are multicopy genes that encode for cathepsin L-like cysteine proteinase B (cpb), a major antigen of *Leishmania* parasites.

PCR RFLP assays targeting cpb genes and their non-coding inter-genic sequences were also developed and applied for characterization of strains from the *L. donovani* complex [83] (Table2). The following enzymes were used for intra-genic cpb PCR-RFLP: *Hinf*I, *Taa*I, *Hae*III, *Cfr*I, *Hpa*II, and *Sdu*I, and for inter-genic cpb PCR-RFLP: *Eam*1104I, *Nsp*I, *Hae*III, *Acy*I, and *Hae*II [83]. The discriminatory power of this assay was compared with that of PCR-RFLP analysis of the gp63 gene, and multilocus enzyme electrophoresis (MLEE). Restriction patterns of the cpb locus were polymorphic, but less so than gp63 patterns and presented differences with MLEE, supporting a different classification of parasites. The applicability of the developed cpb PCR RFLP approach also allowed direct genotyping of parasites in bone marrow aspirates and blood samples obtained from VL patients in Nepal [83]. This cpb PCR RFLP approach, in addition to a gp63 PCR-RFLP analysis, were applied to study 59 isolates of the *L. infantum* species obtained from different regions in Algeria, originating from various clinical forms and hosts, and assigned to different zymodemes [84]. Among the four analyzed zymodemes, 15 different genotypes were obtained. Also, cpb polymorphism showed two interesting trends: a possible relationship with the cutaneous origin of the isolates and an association with a West-East cline [84].

Different species–specific PCR assays were developed using these genes as target. PCR assays discriminating *L. donovani* from *L. infantum* were developed [25,26]. An *L. donovani* species-specific PCR primer pair amplifies a 317bp at the 3' end of cpb gene of *L. donovani* whereas it does not generate an amplicon for *L. infantum* [26]. Another PCR that was developed based on cysteine protease B genes differentiates *L. infantum* from *L. donovani* by their fragment length: a 741bp product (cpbF) characterized *L. donovani* strains, and a 702bp product (cpbE) *L. infantum* strains [25]. This primer pair more recently was tested, in addition to a newly designed one (cpbEF For/L.inf Rev, Table2), on 10 Tunisian *L. infantum* isolates. The amplification showed size polymorphism of cpbEF genes with either a 702bp or a 741bp product, even though the species *L. donovani* has never been described in Tunisia and the Mediterranean region [85].

Five species-specific PCR tests that can discriminate each of the Old World species: *L. infantum*, *L. donovani*, *L. tropica*, *L. aethiopica*, and *L. major* in cultured parasite isolates were also developed [86] (Table2). All the PCRs are based on the species-specific amplification of the cpb genes as each primer pair amplifies only one of the different cpb copies present in a particular species. In addition, the authors established the adaptation of 2 of these assays for oligochromatography detection, which is a rapid dipstick test for visualization of specific amplified *L. infantum* and *L. donovani* products. They concluded to the value of these assays for the identification of parasites *in vitro* but the assays were not shown sensitive enough to identify *Leishmania* parasites within clinical samples [86].

However, upon sequencing of the cpb- coding region in clinical isolates of *L. aethiopica*, specific PCR primers (V5F/V10R) were developed to differentiate this species from *L. tropica*, *L. major*, *L. donovani* and *L. infantum* by direct PCR (Table2). This cpb PCR proved to be sensitive enough to detect *L. aethiopica* from biopsy samples [87].

Recently, primers developed in [25] were used and new ones were designed, to set up three species-specific PCR assays based on the amplification of different copies and parts of the cpb genes (Table2) [85]. They allowed amplification of 1176bp, 600bp and 325bp fragments, thus discriminating between Old World Tunisian *L. major*, *L. tropica* and *L. infantum* species, respectively [85].

Multi-copy cpb genes have been recently used to develop a species–specific *L. infantum* LAMP assay (Loop-Mediated Isothermal reaction) for the diagnosis of canine leishmaniasis in Tunisia [88]. This isothermal nucleic acid amplification technique uses intrinsic properties of the enzyme (*Bst* DNA polymerase) for auto-strand displacement DNA synthesis to amplify large amounts of DNA within 30–60 minutes. The amplification reaction that is conducted at only one temperature does not require a thermocycler and takes profit of the intricate design of a set of six specific primers [89]. LAMP has emerged as a powerful tool for diagnostics and has been successfully developed for several protozoan parasitic diseases including leishmaniasis [90]. Use of cpb genes in the LAMP assay successfully allowed to detect the *L. infantum* DNA with a specific amplification, as no cross reaction was seen, with *L. major*, *L. tropica*, *L. turanica*, *L. aethiopica*, *L. tarentolae*, *L. gerbilli*, *Trypanosoma cruzi*, or human genomic DNA. In addition, LAMP assay showed a higher sensitivity when compared to conventional cpb based PCRs [88].

Cpb coding sequence and UTR targets have a proven and good potential to characterize or identify *Leishmania* species. Their antigenic nature makes them interesting to describe epidemiological features in some areas. However in spite of being multi-copy targets, sensitivity of their detection seem to be limited likely due to sequence variations underlying the primers used.

2.2.7. Cytochrome gene sequencing

Cytochromes are involved in the electron transport process of the mitochondrial respiratory chain. They are considered one of the most useful genes for taxonomy given their slow evolution rate. They were used for discrimination of *Leishmania* parasites as well as for exploring their phylogenetic relationships. Cytochrome oxidase II gene has been first analyzed

for sequence variation in 22 *Leishmania* isolates representative of the *L. donovani* complex from different geographical origins [91]. Phylogenetic analysis produced maximum parsimony, neighbor joining and maximum likelihood trees that were congruent and showed two clades corresponding to the species *L. donovani* and *L. infantum*. Furthermore, the molecular haplotypes were concordant, in general, with the isoenzyme data of the complex [91]. Interestingly, *L. donovani* isolates from Sudan were shown to possess the most ancestral cytochrome oxidase II sequence with a single haplotype that was very close to that of *L. major* [91]. The data provided in this work allowed an approximate dating of the origin of the *L. donovani* complex to a period contemporary to or predating the spread of modern humans out of Africa [91].

Cytochrome b (*Cyt b*) gene has also been used to determine the nucleotide sequence from 13 human-infecting *Leishmania* species from the New and Old Worlds [92]. The phylogenetic relationships based on this gene, showed good agreement with the classification of Lainson & Shaw [93] except for the inclusion of *L. major* in the *L. tropica* complex and the placement of *L. tarentolae* in another genus [92]. The same group has further applied this method to other *Leishmania* species to construct a new phylogenic tree [94]. A total of 30 *Leishmania* and *Endotrypanum* WHO reference strains were analyzed. The phylogenic tree obtained showed mainly the exclusion of *L. major* from the *L. tropica* complex, the placement of *L. tarentolae* in the genus and location of *L. turanica* and *L. arabica* far from human pathogenic *Leishmania* strains [94].

Since that, *Cyt b* gene have been sequenced in several studies and was shown to be able to identify the *Leishmania* species, in Pakistan [95,96], in Colombia [97] and in China [98]. Furthermore, results of *Cyt b* gene sequencing of 69 cutaneous leishmaniasis cases in Pakistan showed that only *L. tropica* was found in highland areas and only *L. major* in lowland areas [96]. Importantly, among *L. major* samples analyzed, three types of *Cyt b* polymorphism were found, including 45 cases of type I, six of type II and one of type III [96]. The authors reported for the first time on the presence of polymorphisms in *L. major* (types I, II and III) based on species identification using *Cyt b* gene sequencing from clinical samples [96].

This target is a slow evolving DNA molecule and is thus considered as a good marker for phylogeny. Being located on the mitochondrial maxicercle, the copy number constitutes another advantage. Given demonstration of natural genetic exchange experimentally [99] and naturally [100], these targets known to have a monoparental transmission (also confirmed for *Leishmania*) could be ideal for genetic exchange analyses.

2.2.8. Other molecular tools

Several other molecular tools have also been used for identification and characterization of *Leishmania*. These include quantitative PCR, AFLP, LAMP assay and others.

In recent years, quantitative PCR methods based either on SYBR Green or TaqMan technology have been set up for the quantification of *Leishmania* in different types of biopsies from mice, dogs and also from human peripheral blood, targeting either single-copy or multi-copy sequences with high sensitivity and reproducibility [101–104]. In particular, quantitative real time PCR assays (qPCR) were developed to detect and rapidly differentiate *Leishmania* species

and also to quantify parasites within clinical samples. Primers used recognized kinetoplast minicircle [105,106] and ribosomal DNA [107].

Amplified fragment length polymorphism (AFLP) has also been developed for *Leishmania* typing [108]. This technique essentially probes the entire genome at random, without prior sequence knowledge. Thus, it is ideally suited as a screening tool for molecular markers linked with biological and clinical traits. It is a PCR-based technique that uses restriction enzymes to digest DNA, followed by ligation of adapters to the ends of the restriction fragments, which will be then amplified using specific primers. The amplified fragments are separated and visualised on denaturing polyacrylamide gels, through autoradiography or fluorescence methodologies or using automated capillary sequencing instruments. AFLP was adapted to the *Leishmania* genome and validated on a panel of samples from the *L. donovani* complex. Results were highly congruent with previous analyses using multiple other molecular tests [109]. AFLPs are particularly useful for assessing genetic variation and genome mapping over other existing molecular techniques (reviewed in [110]).

Assays using alternative amplification technologies such as quantitative nucleic acid sequence-based amplification (QT-NASBA) based on amplification of 18S RNA or Loop mediated isothermal amplification (LAMP) targeting rRNA, kinetoplast DNA or a multigenic family were also tested on *Leishmania* infected samples. QT-NASBA yielded a sensitivity of 97.5% and a specificity of 100% when tested on skin biopsy samples from Old and New World CL patients [111]. A generic loop mediated isothermal amplification (LAMP) of reverse transcribed 18SRNA had a 83% sensitivity on blood samples of VL patients from Sudan and 98% sensitivity on skin biopsies of CL patients from Suriname [90]. An *L. donovani* specific LAMP was developed targeting kinetoplast minicircle DNA that had 80% sensitivity on 10 blood samples of VL patients from Bengladesh [112]. This assay evaluated on a larger number of patients (N=75) and 101 negative controls had 90% sensitivity and 100% specificity; these performances were found comparable to a nested PCR assay tested on the same samples [113]. An *L. infantum* specific LAMP assay, targeting the cysteine protease B multi copy gene was also recently developed [88]. This tool applied on detection of dog infection in Tunisia had a sensitivity of 54% and a specificity of 80%, a better performance than the one obtained with a Cpb PCR assay [88]. LAMP assays constitute promising tools for rapid and sensitive detection of *Leishmania* DNA, however for discrimination of *Leishmania* species and strains other tools may appear superior at this stage. Their main advantage remains the rapid delivery of results and the minimal equipment requirement.

3. Strain typing

3.1. Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) refers to analysis based on the DNA sequence of multiple gene targets. It is based on the comparison of partial sequences (usually 700 bp) of a defined set of housekeeping genes. Similarly to MLEE, alleles are scored as identical or not, regardless of how many different polymorphic loci they have. Strains sharing the same allele combina-

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tions for the set of genes tested are referred to as sequence types. MLST is able to detect co-dominant single nucleotide polymorphisms (SNP) and although indels can complicate the analysis, they are extremely rare in protein-coding genes.

The first *Leishmania* complex that has been studied with MLST is the *L. donovani* complex. Two sets of 5 loci corresponding to genes coding for enzymes used in MLEE were studied: one set with asat, gpi, nh1, nh2 and pgd and the other one with icd, me, mpi, g6pdh, and fh [7,8]. Results were found to be largely in agreement with the results from MLEE although some key discrepancies were found and increased resolution was obtained. Thus silent SNPs were found that provide further resolution, such as a single SNP in gpi that distinguishes between strains of *L. infantum* [7]. However, SNPs responsible for amino acid changes were also found in genes coding for enzymes giving indistinguishable electrophoretic profiles, mainly in nh2, which had the same protein band for all *L. donovani* complex strains. MLST study contributed to better understanding of *L. donovani* complex phylogeny and taxonomical position of the species *L. infantum* and *L. donovani* [114]. It was a strong argument to question the position of *L. archibaldi* as a species [6] and existence of MLEE defined *L. infantum* in Sudan [8]. It also highlighted potential occurrence of genetic exchange among circulating parasites in East Africa [7,8].

MLST using 6 gene targets that are not associated with MLEE analysis (inorganic pyrophosphatase, spermidine synthase 1, hypoxanthine-guanine phosphoribosyl transferase, mitogen-activated protein kinase, RNA polymerase II largest sub-unit and adenylate kinase 2) have been used to characterize suspected *L. major/L. infantum* hybrids and representative co-endemic strains in Portugal [115]. Sequence analyses confirmed MLEE hybrid profiles and hybrid status with occurrence of heterozygous positions in the target genes that so far were not studied for their diversity within *Leishmania* species. In a more recent work, 2 of these genes and 5 others (Elongation initiation factor 2 alpha subunit, zinc binding dehydrogenase-like protein, translation initiation factor alpha subunit, nucleoside hydrolase-like protein and a hypothetical protein located on chromosome 31) were analyzed on a panel of 222 strains representative of 10 different species in 43 countries in Eurasia and Africa, corresponding to 110 zymodemes with the objective to study the genetic diversity of the genus *Leishmania*, improving our knowledge on the genetic structure and genomic evolution mechanisms of this genus [116]. Seven genetically robust clusters were obtained that overlapped with most of the biochemical taxonomy groups: clusters I, III, IV, V and VI included strains belonging to the MLEE-based species *L. aethiopica*, *L. arabica*, *L. turanica*, *L. gerbilli* and *L. major*, respectively and cluster II included the *L. tropica* and *L. killicki* strains; with the exception of the species that cause forms of visceral leishmaniasis (cluster VII that comprised strains from *L. donovani*, *L. infantum* and *L. archibaldi*) in line with the concept of species complex suggested for this group. No observations were made of interspecific recombination or genetic exchange between the different species but these strains were selected for the study as not resulting from a likely genetic exchange [116]. It is anticipated to observe more informative studies increasing the number of markers or the strains circulating within selected endemic areas notably that co-sympatry of multiple parasite species is a well-established feature in many endemic areas.

In the New World, four housekeeping genes (glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), mannose phosphate isomerase (MPI) and isoci-

trate dehydrogenase (ICD)) were sequenced from 96 *Leishmania* (*Viannia*) strains that were chosen to be representative of the zymodeme and geographical species diversity of this subgenus, in South America, and in particular Brazil, in order to assess their discriminatory typing capacity and refine phylogeny of the *L.* (*Viannia*) species [117]. A large number of haplotypes were detected for each marker. Maximum parsimony-based haplotype networks showed separated clusters in each network, corresponding to strains of different species, congruent with the MLEE identification. Besides, NeighborNet formed by the concatenated sequences confirmed species-specific clusters. This analysis also suggested recombination occurring in *L. braziliensis* and *L. guyanensis*. However, using phylogenetic analysis, the species *L. lainsoni* and *L. naiffi* were shown to be the most divergent species and placed the *L. shawi* species in the *L. guyanensis* cluster, not as a distinct species. The authors also found the *L. braziliensis* strains to correspond to one widely geographically distributed clonal complex in Brazil and another restricted to one endemic area, in a region bordering Peru [117].

The main advantage of MLST is the possibility of generating genus-wide phylogenies, since MLST markers are co-dominant and are amenable for population and phylogenetic analyses. Also, given the high quality of sequence data, results can be easily compared between laboratories. Compared to MLEE, MLST does not necessarily require sterile culture of parasites. In addition, simultaneous typing of reference strains and sequencing can be done commercially without in-house specialized equipment. For those reasons, MLST is likely to become the gold standard basis for taxonomy and thus identification of *Leishmania*. One expected drawback could be the inherent limit of detection of nucleotide allelic diversity associated to direct sequencing of PCR products, which could be overcome by more lengthy analyses like cloning of parasites or PCR products. One consequence of this drawback is that MLST should not be considered as typing tool but an analysis tool. Another application could be diagnosis as recently new species-specific genetic polymorphisms were identified in the genes that confer the phenotypic variations in the MLEE assay [118]. Indeed, sequencing of the MPI and 6PGD genes was sufficient to differentiate among closely related species causing New World leishmaniasis, in Peru. The same group took advantage of these polymorphisms and designed a new real-time PCR assay based on FRET (fluorescence resonance energy transfer) technology and melting curve analysis using SYBR green. The assay was highly sensitive and correctly identified each of the five main species that cause tegumentary leishmaniasis in the New World, directly from clinical samples [119].

3.2. Multilocus microsatellite typing (MLMT)

Microsatellites are repeated motives of 1–6 nucleotide(s), which present allelic length variation. They mutate fast, therefore, 10–20 independent markers have to be analyzed for each strain owing to homoplasy. Microsatellite sequence variation results from the gain and loss of repeat units, which can easily be detected after amplification with specific primers annealing to their flanking regions. Then length polymorphisms are detected using PAGE, MetaPhor agarose gel electrophoresis or, preferably, automated capillary sequencers. A multilocus microsatellite profile is compiled for each sample from the fragment length measured for the microsatellite markers analyzed.

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During the last years, microsatellite-based approaches have been developed for strain typing within the genus *Leishmania* to overcome the lack of discriminatory power of MLEE and other molecular tools. So far, microsatellite loci with high discriminatory power and suitable for characterizing closely related strains have been published for the *L. donovani*/*L. infantum* complex [120–122], *L. major* [123,124], *L. tropica* [125] and for species of the subgenus *L.* (*Viannia*) [126–128].

3.2.1. Subgenus L. Leishmania

3.2.1.1. L. donovani complex

Within the *L. donovani* complex, a set of 15 microsatellite markers have been applied to type strains of *L. donovani* and *L. infantum* isolated from the main endemic regions for VL (India, East Africa, Mediterranean region, Asia and South America) [129]. Six principal genetically distinct populations were identified: 2 populations of *L. infantum* from the Mediterranean area and South America comprising the MON-1 and non-MON-1 strains, respectively; 2 populations of *L. donovani* from Sudan and Ethiopia; 1 of *L. donovani* MON-2 from India; and 1 consisting of strains of *L. donovani* (MON-36, 37 and 38) from Kenya and India. These results corroborated the fragmentary data published in numerous studies using other genetic markers. Interestingly, the highest microsatellite diversity was observed for *L. infantum* from the Mediterranean basin and the lowest for *L. donovani* from India. Using 34 additional microsatellite sequences, analysis showed the homogeneity of *L. donovani* from the Indian subcontinent [130].

Different genetic groups of strains of *L. infantum* were also observed when strains from Algeria, Tunisia, the Palestinian Authority and Israel were subjected to MLMT. Microsatellite typing of strains belonging to zymodemes MON-1, MON-24 and MON-80 identified 3 different populations in Algeria and in Tunisia [131,132]. The MON-1 strains were assigned to 2 different populations one of which contained only local strains and the other local and European strains of MON-1. The non-MON-1 strains were always separated from the MON-1 ones. Gene flow was detected between the two MON-1 populations and the local MON-1 and the non-MON-1 populations, respectively [131,132]. *L. infantum* Israeli and Palestinian strains obtained from infected dogs and human cases showed 2 main populations genetically different from European populations, one of which is sub-divided in geographically distributed sub populations [133].

In Spain, *L. infantum* strains from a rural leishmaniasis-endemic area, from which 94 were obtained from dogs, 15 from sand flies, and 1 from a human visceral case, were MLMT studied [134]. Results showed existence of 17 genotypes that were detected using 10 microsatellite markers belonging to 3 different targets. They also showed the heterogeneous distribution of *L. infantum* species in hosts living in sympatric conditions.

Analysis of *L. infantum* strains having a New World origin by MLMT indicated that these strains were more similar to MON-1 and non-MON-1 sub-populations of *L. infantum* from southwest Europe, than to any other Old World sub-population [135] thus indicating that the parasite has been recently imported multiple times to the New World from southwest Europe.

Within the *L. donovani* complex, *L. donovani*, *L. infantum* and *L. archibaldi* strains from Sudan were studied by MLMT technique [6]. The authors found one single monophyletic *L. donovani* clade and argued that the isoenzyme differentiation of *L. donovani* and *L. infantum* in East Africa was misleading and that *L. archibaldi* is an invalid taxon [6].

Analysis of *L. donovani* strains from India, Bangladesh, Sri Lanka and Nepal showed that in Sri Lanka the causative agent of CL is most closely related to parasites causing VL in India [136] and that genetically homogeneous strains are circulating in the Indian subcontinent [130]. On the other hand, *L. donovani* strains belonging to the MON-37 zymodeme and originating from different geographical origins (India, Sri Lanka, Middle East, Cyprus and East Africa) were MLMT analyzed [9]. Zymodeme MON-37 was found to be paraphyletic, representing different genetic groups corresponding to their geographical origin and strains from Cyprus were clearly different from all others and could be autochthonous [9].

3.2.1.2. L. tropica

MLMT technique was also applied for *L. tropica* strain typing. Indeed, 117 strains from Asia and Africa were used and revealed 10 genetic groups, which were largely correlated to the geographical origin of the strains [125]. Different genetic groups were shown to co-exist in strains from the Middle East and Morocco. However, the authors postulated that recent spread of new genotypes has occurred recently in the Middle East and suspected an African origin of the *L. tropica* species [125].

3.2.1.3. L. major

Concerning *L. major*, 106 strains from Central Asia, Africa and the Middle East were analyzed using MLMT, based on 10 different microsatellite markers [124]. The study showed three main populations corresponding to the three geographical regions studied that were further subdivided into 2 sub-populations. Interestingly, the African and Middle Eastern populations seemed to be more genetically diversified than the Central Asian population [124].

3.2.2. Subgenus L. Viannia

Within the New World *L. Viannia* subgenus, the first MLMT studied species were *L. braziliensis* and *L. peruviana*. Fifty- nine analyzed Peruvian strains showed emergence of multiple *L. braziliensis*/*L. peruviana* hybrids [137]. Then, 124 *L. braziliensis* strains from Peru and Bolivia were investigated for their genetic polymorphism at 12 microsatellite loci [127,138]. A substantial genetic diversity with high levels of inbreeding, inconsistent with a strictly clonal reproduction was shown. Besides, a large genetic heterogeneity between populations within countries was described, which evidenced a strong population structure at a microgeographic scale [138].

In another study, polymorphisms of 30 strains of *L. braziliensis*, 21 strains of *L. guyanensis*, and 2 strains of *L. peruviana* from Brazil, Paraguay and Peru were analyzed at 15 independent microsatellite loci [128]. All strains except two *L. guyanensis* had individual MLMT types. In addition, three main clades were found, that consisted of one population of strains of *L.*

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guyanensis only, another one with strains of *L. braziliensis* from Paraguay and Brazil, and the last one with strains of *L. braziliensis* and *L. peruviana* [128].

Recently, 28 strains of the main species of the *L. guyanensis* complex (*L. guyanensis* and *L. panamensis*), collected in Ecuador and Peru were investigated in an MLMT study, with 12 microsatellite markers [139]. An important heterozygote deficit was observed in these populations, similar to the previously reported results in *L. braziliensis* complex [138]. They further showed genetic polymorphism and geographical differentiation on the *L. guyanensis* complex [139].

All together, these studies confirmed that microsatellite markers constitute good tools for typing and population genetic studies of *Leishmania*. Their additional advantage resides in the possibility of their use directly in biological material without culturing of parasites [130,140]. Moreover, accurate, quality controlled microsatellite profiles could be stored in databases and compared between different laboratories.

4. *Leishmania* parasite evolution, genetics and genome analyses – Consequences and prospects

For many years *Leishmania* parasites have been considered to replicate clonally, without genetic exchange. Indeed, Tibayrenc proposed that clonal evolution in micropathogens be defined as restrained recombination on an evolutionary scale, with genetic exchange scarce enough to not break the prevalent pattern of clonal population structure (Reviewed in [141,142]). The two main manifestations of clonal evolution are strong linkage disequilibrium (LD) and widespread genetic clustering ("near-clading"). These authors hypothesized that this pattern is not mainly due to natural selection, but would originate chiefly from in-built genetic properties of pathogens, that would allow like for other microorganisms (viruses, bacteria, protozoan parasites) to keep a balance between clonality and recombination, which would help escape from recombinational load. This way, to face evolutionary challenges, pathogens would be equipped with "clonality/sexuality machinery" that would function as alternative allelic systems [141,142]. However, an accumulation of molecular evidence indicates that there are inter-specific [115,137,143–146] and intra-specific [132,138] hybrids among natural populations. Genetic exchange was finally demonstrated experimentally in 2009 [99]. In fact, double drug resistant *Leishmania* major hybrids were produced by co-infecting *Phlebotomus duboscqi* (a natural *L. major* vector) sand flies with two strains carrying different drug resistance markers. The nuclear genotypes were consistent with a Mendelian transmission leading to a heterozygous first generation progeny [99]. The anticipated continuity of these studies was to co-infect sand flies with transgenic *Leishmania* carrying two different markers that are fluorescent, in an attempt to visualize the recombination events microscopically [147]. In 2011, for the first time, using a fluorescent protein detection system to observe yellow hybrid promastigotes in *Phlebotomus perniciosus* and *Lutzomyia longipalpis* midguts, *L. donovani* hybrids were observed, 2 days post bloodmeal, and the morphological stages involved were found to be short procyclic promastigotes [100]. However, the parasites could not be recovered and propagated to confirm

their hybrid genotypes [100]. Recently, the analysis of the mating competency of *L. major* strains have been expanded to include pairwise matings of multiple isolates bearing independent drug markers [148]. Also, the timing of the appearance of hybrids and their developmental stage associations within both natural (*Phlebotomus duboscqi*) and unnatural (*Lutzomyia longipalpis*) sand fly vectors was followed. Genotype analysis of a large number of progeny clones showed a chromosomal inheritance of both parental alleles at 4–6 unlinked nuclear loci, consistent with a meiotic process, and a uniparental inheritance of kinetoplast DNA [148]. A low frequency of nuclear loci showed only one parental allele, suggesting loss of heterozygosity, most likely arising from aneuploidy, which is common in *Leishmania*. In the natural vector, when comparing the timing of hybrid formation and the presence of developmental stages, the authors suggested that nectomonad promastigotes are the most likely mating competent forms, with hybrids emerging before the first appearance of metacyclic promastigotes [148].

MLMT analysis showed that recombination events are much more frequent in *Leishmania* than previously thought. Indeed, MLMT analysis of Bolivian and Peruvian *L. braziliensis* showed frequent sexual crosses of individuals from the same strain (inbreeding) [138]. The substantial heterozygote deficiency and extreme inbreeding found in this study is not consistent with a strictly clonal reproduction. The authors came to the conclusion that *Leishmania* parasites may alternate between clonal and sexual modes of reproduction, occurring most probably in the vector [138]. Sexual fusion may frequently take place between genetically related parasites or even within the same strain with occasional recombination events between individuals of different genotypes.

Also, *L. braziliensis*/*L. peruviana* hybrids were found to be quite common in a Peruvian focus where both species can occur sympatrically [137]. In the Old World, natural *L. infantum*/*L. major* hybrids were experimentally transmitted by *Ph. papatasi*, usually only competent to transmit *L. major* [149]. This suggests that hybrids may circulate using this sand fly vector and spread into new foci throughout the broad range of *Ph. papatasi* distribution.

The fact that *Leishmania* can undergo genetic exchange is potentially of profound epidemiological significance since this could facilitate the emergence and spread of new genotypes and phenotypic traits. Also, hybrid offspring might show a strong selective advantage relative to the parental strains. In [149], the authors have shown that natural hybrids could have enhanced transmission potential and a positively affected fitness.

New high-throughput sequencing technologies have opened the door for population genome analyses and genome-wide association studies. Genome of the *L. major* species was the first to be fully sequenced [150] followed by *L. infantum* and *L. braziliensis* [151]. Comparison of the three genomes revealed conservation of synteny and identified only 200 genes having a differential distribution between the three species. Such genes may encode for proteins implicated in host-pathogen interactions and parasite survival in the macrophage [151]. The species *L. mexicana* and *L. donovani* were subsequently sequenced [152,153] and the reference genomes for *L. major*, *L. infantum*, and *L. braziliensis* were refined [152]. This has allowed the identification of a remarkably low number of genes or paralog groups unique to each of the species *L. mexicana, L. major, L. infantum*, and *L. braziliensis* (2, 14, 19, and 67, respectively). Besides, *L. major* and *L. infantum* were found to have a surprisingly low number of predicted

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heterozygous SNPs compared with *L. braziliensis* and *L. mexicana*. Chromosome copy number also varied significantly between species, with nine supernumerary chromosomes in *L. infantum*, four in *L. mexicana*, two in *L. braziliensis*, and one in *L. major*. The authors also showed that gene duplication events occur more frequently on disomic chromosomes [152]. In addition to sequencing of an *L. donovani* reference genome, a recent study also included sequence analysis of a set of 16 related clinical lines, isolated from VL patients in Nepal and India, which also differ in their *in vitro* drug response [153]. Sequence comparisons with other *Leishmania* species and analysis of single-nucleotide diversity showed evidence of selection acting on different surface- and transport-related genes, including genes associated with drug resistance. Extensive variation in chromosome copy number between the analyzed lines was also shown. In association to drug resistance, they also showed structural variation, including gene dosage and copy number variation of a circular episome, present in all lines [153].

Genomic research on *Leishmania* is taking promising directions, mainly upon sequencing of the main pathogenic species [150–153] and also the non pathogenic *L. tarentolae* [154] which will enable to answer key questions on population genetics and ultimately unravel many important aspects related to drug resistance and virulence, which are especially relevant for control of the disease.

Novel genomics technologies are expected to bring more powerful tools to characterize the pathogens and particularly the infectious stages of *Leishmania* parasites. It will be particularly useful to fully characterize the parasites within the lesions/hosts in their microenvironment. While so far expression profiling relied mainly on microarray analysis which revealed only a limited number of differentially expressed genes across developmental stages [155], or species [156]. RNA sequencing technology seems very promising to highlight transcriptional events that are associated to parasite life cycle, infection or pathology. Previous studies have demonstrated a correlation between gene expression and gene copy number [157,158]. It was further hypothesized that "Increased gene copy number due to chromosome amplification may contribute to alterations in gene expression in response to environmental conditions in the host, providing a genetic basis for disease tropism" [152]. Other studies have also suggested that *Leishmania* parasites do not respond dynamically to host immune pressure, and that any influence of varying transcript levels on virulence and pathogenicity of the different *Leishmania* species is likely to result from the differential expression of conserved genes between species and/or the expression of a small number of genes that are differentially distributed between species [159].

Genome-wide multilocus genotyping in malaria research through novel sequencing technologies has allowed the identification of almost 47000 single nucleotide polymorphisms (SNPs) across the *Plasmodium* genome [160]. This allowed development of microarray–based platforms for screening more than 3000 SNPs that were successfully applied for population genetic analyses and genome-wide association studies in *P. falciparum* [161,162]. Similar studies still need to be developed for *Leishmania*.

5. Conclusion

Epidemiological, taxonomic and population genetic studies of *Leishmania* require good sampling methods and appropriate molecular markers that allow discrimination at different levels. Answering key epidemiological questions requires new or improved tools that allow discrimination of *Leishmania* parasites at different levels. The MLEE, considered as gold standard technique, needs cultured parasites and lacks discriminatory power. PCR assays are likely to replace isoenzyme analysis since they enable direct detection and identification of different *Leishmania* species in human and animal samples and also in infected sand flies. Many of the PCR assays described in the literature have proven useful in numerous field studies. However, they still need to be standardized and validated as diagnostic PCR assays and comparisons of the sensitivity and specificity parameters of the different approaches need also validation under routine conditions. In general, more than one assay is necessary to obtain fully satisfactory analysis of field samples. Given emergence context and changing eco-epidemiological trends, multiple tools will be needed to fully investigate the transmitted parasites.

At the strain level differentiation, MLMT has potential for being a gold standard, because on its principle it is expected to be reproducible and brings possibility of data storage and exchange. However, microsatellite markers are largely species-specific in *Leishmania* and different marker sets have to be used according to species. Such databases do not exist yet and data generation will need standardization. It may also require access to automated sequencers and good knowledge of population genetics programs. On the other hand MLST appears potentially as more powerful for phylogenetic and evolutionary studies although less discriminatory than MLMT. It is most probably this technique that will advantageously replace MLEE in the future. Some results showed that the same targets could be used across the *Leishmania* genus, which will enable comparisons of distances between the species but also of the degree of genetic diversity within species [163]. Here also it will require access to automated sequencers and adequate analytical programs. Cost of both approaches are relatively high and inherent limitations will be most likely overcome by the next generation sequencing approaches expected to gain momentum in a near future. *Leishmania* population genomics still needs to be developed and made accessible to researchers in disease endemic countries to best achieve its public health potential.

Parasite knowledge is so far built on strains obtained *in vitro*. Increasing interest in *Leishmania* parasite analyses will likely identify novel genotypes or organisms, a challenge for our current knowledge on parasite taxonomy and assays to identify and characterize parasites. Improving ways to enhance knowledge on parasites within samples remains a priority.

In spite of the increasing potential of sophisticated technologies and techniques, some disease endemic areas still need simple assays for eco-epidemiological investigations or diagnosis as well as capacity building in this highly relevant area to disease control.

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