Review

Leishmaniases in the Mediterranean in the era of molecular epidemiology

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Molecular tools are used increasingly for descriptive epidemiological studies in different Mediterranean foci of visceral and cutaneous leishmaniases. Several molecular markers with different resolution levels have been developed to address key epidemiological questions related to the (re-)emergence and spread of leishmaniases, as well as its risk factors: environmental changes, immunosuppression and treatment failure. Typing and analytical tools are improving but are not yet addressing all epidemiological issues satisfactorily. There is an urgent need for better cooperation between laboratory scientists and epidemiologists and for regional epidemiological surveillance of these infectious diseases that affect all Mediterranean countries.

Mare nostrum: a dynamic communication hub

For centuries, the Mediterranean Sea (called by the Romans *mare nostrum*, i.e. 'our sea') has functioned as a major communication and exchange platform, providing a dynamic compromise between political unity and cultural diversity in the region. This probably influenced the historical and epidemiological pattern of many infectious diseases, among them leishmaniases. Lesions suggestive of cutaneous leishmaniasis (CL) have been known in Egypt since 2000 BC [1] and in Assyria since 650 BC [2] and were recorded, among other places, in Crete and Algeria in the 18th century [2]. Evidence for visceral leishmaniasis (VL) has been found recently in ancient Egyptian mummies from the Middle Kingdom period (2050–1650 BC) [3]. Nowadays, the two forms of the disease are endemic in Mediterranean countries and are essentially caused by three parasite species: *Leishmania infantum* (zoonotic VL and CL), *Leishmania tropica* (anthroponotic and zoonotic CL) and *Leishmania major* (zoonotic CL). The epidemiology of leishmaniases is rapidly evolving around the Mediterranean basin, as in other regions of the world, because of three major risk factors: (i) environmental changes; (ii) immunosuppression and (iii) treatment failure. A multidisciplinary network (LeishMed) has been launched to document the importance of these main risk factors in the spread of CL and VL around the

Glossary

Clade: Defined as a taxonomic group with a single common ancestor.
DNA fingerprinting: Based on hybridization of non-specific probes to genomic DNA fragments obtained after digestion with restriction endonucleases. Restriction fragments are separated by polyacrylamide gel electrophoresis (PAGE) or on agarose gels and blotted onto a membrane before hybridization. The resulting banding patterns are transformed into presence or absence matrices and analyzed by different phylogenetic methods.
***Leishmania* species**: Defined by a numerical taxonomy approach based on the results of MLEE (phenetic species).
***Leishmania* strain**: Defined here as parasites present in one original isolate, not cultured but analyzed directly in the biological material taken; or a set of laboratory replicates from the same original isolate (stock).
Multilocus enzyme electrophoresis (MLEE): Standard MON classification [6] is based on 15 enzymatic systems analyzed by starch gel electrophoresis. The same enzyme from different samples might show different mobility in the gel (isoenzyme or electromorphs; strictly allozymes). Zymodemes are strains sharing the same isoenzyme profiles for all 15 enzymes. MLEE markers are co-dominant.
Multilocus microsatellite typing (MLMT): Microsatellites are stretches of short nucleotide motives (1–6 bp) that are repeated tandemly, which present allelic length variation. They mutate fast, however, 10–20 independent markers have to be analyzed for each strain owing to homoplasy. Microsatellite-containing sequences are amplified and length polymorphisms are detected using PAGE, MetaPhor agarose gel electrophoresis or, preferably, capillary sequencers. A multilocus microsatellite profile is compiled for each sample from the fragment length measured for the microsatellite markers used and analyzed directly or after the calculation of genetic distances using different types of population genetic analyses. Microsatellite markers are co-dominant.
Multilocus sequence typing (MLST): A highly standardized method that relies on the comparison of partial sequences (usually 700 bp) of a defined set of housekeeping genes (7–10). Similarly to MLEE, alleles are scored as identical or not, regardless of how many different polymorphic loci they have. Strains sharing the same allele combinations for the set of genes tested are referred to as sequence types. MLST markers are co-dominant and are amenable for population and phylogenetic analyses. Alternatively, the data can be analyzed as sequences.
PCR fingerprinting: Based on the amplification of genomic DNA with single non-specific primers that anneal to mini- and micro-satellite DNA sequences. The polymorphic DNA fragments are separated on agarose gels and the resulting banding patterns are analyzed as described for DNA fingerprinting.
PCR-RFLP: Different amplified targets are digested with a defined set of restriction enzymes. The restriction fragments are separated by PAGE or on agarose gels. The evaluation of the resulting banding patterns is as for DNA fingerprinting.
Randomly amplified polymorphic DNA (RAPD): Uses a set of single and short random primers for amplifying genomic DNA and generating polymorphic banding patterns. The polymorphic DNA fragments are separated on agarose gels and the resulting banding patterns are analyzed as described for DNA fingerprinting.
Strain tracking: A process capable of identifying the ultimate origin of parasite isolates, for example, by determining the origin of imported cases or sources of infection. It requires a high resolution molecular-typing method, such as kDNA fingerprinting or MLMT, to distinguish between closely related strains.

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Box 1. Phylogenetic and population genetic analyses in molecular epidemiological studies

Phylogenetic analyses present information on the history of populations or organisms in the form of trees. Population genetic analyses provide a snapshot of the current structure of genetic variation within and between populations. Both types of analyses usually rely on neutral markers. Non-neutral genes could, in some situations, be more interesting because they could be under selection or might be a marker for a useful trait. Markers should always be tested for neutrality and chosen based on the question being asked (Table 1). Population genetic studies in diploid organisms rely on co-dominant loci being able to detect all three combinations of alleles possible in a diploid.

Other important requirements that markers should have for molecular epidemiological studies of pathogens are:

- Appropriate discriminatory power for the question addressed
- Stability during *in vitro* or *in vivo* passages
- Reproducibility of results, storable in databases and comparable among laboratories
- They should enable testing assumptions, comparing results from different methods of analysis and examining robustness of results by using re-sampling techniques
- Direct testing on clinical samples
- Simple and easy to perform with satisfying cost–effectiveness

How many markers are needed for molecular epidemiological studies depends largely on the mode of reproduction. In clonal organisms, in which the degree of linkage between loci is high, single markers of sufficient variability might, in theory, prove useful for answering particular epidemiological questions. However, in recombining organisms, each locus might have a separate evolution and a larger set of independent markers is required for detection of the level of variation within a given species. The number of markers needed also depends on the diversity at the loci and the level of genetic differentiation. Studies on intraspecific genetic differentiation should always maximize the number of loci as well as the number of alleles per locus. In addition, the number of markers will be chosen according to the size and quality of the sample. Sample size is crucial because it has a direct effect on the ability to test hypotheses.

Mediterranean and to promote transborder control strategies [4]. One activity of the consortium concerns the application of molecular biology for addressing epidemiological questions that cannot be answered readily by conventional methods. This is reviewed here, with a specific focus on the parasitological aspects of Mediterranean leishmaniases as a model for other epidemiological settings in the world.

Molecular epidemiology

Molecular epidemiology is a commonly (ab)used term for descriptive molecular studies of pathogen populations. Most published studies are based on empirical analyses and, unfortunately, phylogenetic or population genetic approaches to data analysis (Box 1) have rarely been used to date [5]. However, the first aim of molecular epidemiology should be to support studies on the distribution, determinants and control of leishmaniasis in human (or veterinary) populations. Sound molecular epidemiology requires an interaction among different experts: clinicians, veterinarians, epidemiologists, molecular biologists and public-health authorities. This discipline has indeed the potential to advance research on disease causation by analyzing, in parallel, virulence markers of the pathogen and susceptibility markers of the host. Molecular epidemiology approaches can also contribute significantly to disease control when they are used to: (i) identify the species in an outbreak investigation; (ii) study the prevalence of infection in a population; (iii) aid clinical diagnosis and case management; (iv) determine the population structure and the extent of migration of pathogens or vectors; and (v) study the emergence and spreading of drug resistance. Molecular markers depend on accurate knowledge of the predominating mode of reproduction (i.e. clonality versus genetic recombination). Genetic recombination would result in unstable multilocus genotypes, in which case only individual genes could be used for epidemiological tracking [5]. Moreover, molecular tools enable better characterization of insect vectors of leishmaniasis, particularly in defining their populations and detecting and monitoring the dispersion of insecticide resistance. For Mediterranean leishmaniases, specific questions related to the three main risk factors that are being addressed currently using molecular tools are listed in Table 1.

The main challenges for molecular epidemiology in infectious disease are the difficult communication between molecular and non-molecular experts, appropriate sampling and standardization of molecular methods, analytical tools and databases. The need for multidisciplinary communication is often underestimated and the use of sophisticated laboratory markers should not blind researchers to fundamental issues, such as validity and precision. Several authors have warned against suboptimal sensitivity and even specificity of PCR in clinical diagnosis. Because samples for PCR are usually obtained in referral centers, they are prone to selection biases and rarely represent all infected persons at the community level. Molecular assays should be evaluated with the same rigor as other diagnostic tests; proper sample size calculation is mandatory in protocol design, selection and observation biases should be avoided and the multicausal nature of disease phenomena should be accounted for in data analysis. Sampling is often problematic because pathogens are usually difficult to isolate and virulent strains are often over-represented in sample collections, hence the use of direct applications in host tissues should be preferred. A large number of molecular methods are available for the characterization of *Leishmania* from genus to strain levels (Box 2), although most of them lack standardization. Analytical tools are improving, however, they still suffer from major weaknesses, such as: (i) population genetic interpretation (heterozygosity and recombination are still not resolved adequately for organisms without a haploid life stage); and (ii) the integration of molecular and epidemiological data represents a challenge. Widely accessible and compatible databases are fundamental for the integration of data collected by different researchers. Databases need to be linked to collections of reference strains and should be geo-referenced.

Current status of *Leishmania* classification

A major conceptual problem in molecular epidemiology of leishmaniasis is the taxonomy of the etiological agent. Since the first description of the genus *Leishmania* in 1903, the number of species has varied. Multilocus enzyme

Table 1. *Leishmania* epidemiological questions that could be addressed by molecular epidemiology.

Epidemiological issues related to leishmaniases	Molecular identification expected to levels of	Related risk factor(s)[a]		
		Anthropogenic and environmental changes	Immunosuppression	Treatment failure
Confirmation of diagnosis	Genus	✓	✓	✓
Parasitic load in mammal hosts and insect vectors in versatile ecological settings		✓	-	-
Monitoring the efficacy of drug treatment		-	-	✓
Identification of new non-human reservoir hosts (domestic or sylvatic)	Species	✓	-	-
Incrimination of new phlebotomine vectors		✓	-	-
Identification of species associated with treatment failure		-	-	✓
Detection and characterization of new parasite–insect host combination		✓	-	-
Species (*L. aethiopica?*) leading to unique immune anergy		-	✓	-
Introduction of new parasite genotypes (related to fitness in both the mammal and the insect hosts)	Clade	✓	-	-
Colonization of new insect host-vector species by new parasite genotypes		✓	-	-
Different populations of parasites at the geographical level		✓	-	-
Different parasite genotypes present in immunocompromised and immunocompetent individuals		-	✓	-
Particular parasite genotypes related to drug resistance		-	-	✓
Origins of new genotypes	Strain	✓	✓	✓
Differences in gene pools in human and animal hosts		✓	-	-
Detection of epidemics		✓	-	-
Identification of sources of *Leishmania* (surveillance)		✓	✓	-
Identification of sources of resistant strains (surveillance)		-	-	✓
Existence of gene flow between populations owing to human or non-human reservoir migrations		✓	-	-
Relapses owing to recrudescence or *Leishmania* re-inoculation		-	✓	✓
Emergence of drug resistance owing to clonal expansion of resistant parasites		-	-	✓
Emergence of insecticide-resistant insect vector(s)		✓	-	-

[a]Whether these questions can be addressed by using molecular tools for identification of *Leishmania* and sand-fly vectors at different taxonomic levels (✓ indicates yes; - indicates no).

electrophoresis (MLEE) (see Glossary) remains the reference technique for *Leishmania* identification at species and intra-species level. Compared with all other methods, it has been applied to the most varied and largest number of *Leishmania* isolates in the past 25 years. The application of numerical taxonomy and cladistic techniques to electrophoretic data resulted in a classification system [6] compiled in Box 3. Nucleotide divergence began being estimated directly in the early 1970s. Phylogenies based on nucleotide polymorphisms in different genomic targets [7,8] have largely confirmed the taxonomy of the genus *Leishmania* by MLEE, with some exceptions, such as two species endemic in the Mediterranean region. Using MLEE, *L. infantum* (the etiological agent of VL in the region) was thought to exist in Sudan, together with two other viscerotropic species, *Leishmania donovani* and *Leishmania archibaldi* [6,9]. However, recent studies based on many different molecular markers point to the existence of only one visceralizing species in Sudan, *L. donovani*, which is different genetically from Indian and Kenyan *L. donovani* and Mediterranean and South American *L. infantum* [10]. Another discrepancy concerns the status of *Leishmania killicki,* which has been reported increasingly to cause CL in Tunisia and Libya [11,12]. Classified as a separate species by MLEE [6], *L. killicki* was shown to be *L. tropica* by molecular analyses [13].

For classification below the species level, standard MLEE is of limited usefulness. For instance, most of the parasites causing VL in the Mediterranean and in Latin America belong to the zymodeme (MLEE-type) MON-1, making strain tracking within these populations impossible. By using a modified system for isoenzyme typing, genetic heterogeneity could be demonstrated for MON-1 strains [14]. This approach, however, has never been validated on a sufficiently large sample of *L. infantum*. DNA-based methods, such as PCR-restriction fragment length polymorphism (RFLP) of kinetoplast minicircle DNA and multilocus microsatellite typing (MLMT), have high discriminatory power that enables further characterization of the parasite diversity and establishment of genetic links among remote populations [15–18]. These methods might be useful for reconstituting the history of leishmaniasis around the Mediterranean and monitoring its further evolution. They are, however, of limited use for studies above species level.

Effect of environmental changes

The distribution of leishmaniases can be affected greatly by environmental changes as a result of climatic modifications associated with global warming and also as a result of human behavioral factors. Highly discriminatory molecular epidemiology tools applied to both parasite and vector populations might have a major role in monitoring and predicting increase and spreading of the diseases.

As a consequence of global warming, leishmaniases are likely to spread into currently temperate zones, where

Box 2. Molecular tools for epidemiological studies in leishmaniasis

Differentiation at the genus level

This is based on the amplification of kinetoplast minicircle DNA and of variable sequences of the *ssu rRNA* gene (for comparisons, see Refs [55,59,60]). These targets have also been chosen for the development of real-time PCR assays [61,62], enabling determination of parasitic burden in epidemiological studies.

Differentiation at species level

This is achieved by multilocus enzyme electrophoresis (MLEE) or PCR assays.

MLEE is performed only in specialized laboratories, is time-consuming and requires bulk cultivation of parasites. Because MLEE detects differences in protein mobility, allozymes that have indistinguishable mobility will not be identified.

PCR can use species-specific primers [63,64] for diagnosis in endemic areas with only one species present or for reservoir and vector studies. When genus-specific primers are used, species identification is achieved by restriction-fragment length polymorphism (RFLP) or sequence analyses of amplified multicopy genes or intergenic spacers [55,65,66]. This approach has advantages in areas endemic for different species of *Leishmania* and for the identification of imported species.

Differentiation at the strain level

This is achieved by MLEE or by different DNA-based techniques.

DNA-based markers used for molecular epidemiological studies of leishmaniases include:

- DNA and PCR fingerprinting and randomly amplified polymorphic DNA (RAPD) techniques, which require cultured parasites because probes and primers used are not specific for *Leishmania.* DNA fingerprinting with human multilocus minisatellite probe 33.15 was applied to follow an outbreak of VL in central Israel [29]. PCR fingerprinting and RAPD techniques have been applied successfully to characterize *L. tropica* strains in a new Israeli focus [28] as well as to detect intraspecies variation in the *L. donovani* complex [14].
- PCR-RFLP based on sequence polymorphisms in multigene families, such as cysteine protease B and major surface glycoprotein GP63, in intergenic spacers, such as ribosomal internal transcribed spacer, in miniexon sequences [67,68] and in kinetoplast minicircles [15–17]. Numerous studies have used this approach for epidemiological studies in leishmaniasis; those cited here just serve as examples.
- Multilocus microsatellite typing (MLMT) based on length variations measured for 10–20 simple repeats has been evaluated for strain typing and population studies in *L. tropica* and the *L. donovani* complex [13,18,69,70] In *Leishmania,* microsatellite markers are largely species specific and different marker sets have to be developed. MLMT is not suited for inferring phylogeny.
- Multilocus sequence typing (MLST) is, currently, the most powerful technique for phylogenetic studies in *Leishmania.* Different enzymatic systems have detected a considerable degree of heterozygosity in the *L. donovani* complex, genome mosaics and hybrids across different groups of this complex [56,57], but also between *L. infantum* and *L. major* [58], suggesting significant genetic exchange.

These different tools are compared in Table 2.

Box 3. Simplified classification of the genus *Leishmania* based on multilocus enzyme electrophoresis, considering only species pathogenic to humans

The genus *Leishmania* [Ross (1903)] consists of the two subgenera: *Leishmania* [Saf'janova (1982)] and *Viannia* [Lainson and Shaw (1987)].

Six Old World (OW) and two New World (NW) species complexes belong to the subgenus *Leishmania*:

- *L. donovani* complex (OW) with the species *L. donovani* [Layeran and Mesnil (1903)] and *L. archibaldi* [Castellani and Chalmers (1919)]
- *L. infantum* complex (OW) with *L. infantum* [Nicolle (1908)] [also *L. chagasi* Cunha and Chagas (1937)]
- *L. tropica* complex (OW) with *L. tropica* [Wright (1903)]
- *L. killicki* complex (OW) with *L. killicki* [Rioux, Lanotte and Pratlong (1986)]
- *L. aethiopica* complex (OW) with *L. aethiopica* [Bray, Ashford and Bray (1973)]
- *L. major complex* (OW) with *L. major* [Yakimoff and Shokhor (1914)]
- *L. mexicana* complex (NW) with *L. mexicana* [Biagi (1953)] [also *L. pifanoi,* Medina and Romero (1959)]
- *L. amazonensis* complex (NW) with the species *L. amazonensis* [Lainson and Shaw (1972)] [syn. *L. garnhami,* Scorza *et al.* (1979)] and *L. aristidesi* [Lainson and Shaw (1979)]

The species status of the taxa *L. archibaldi* [10] and *L. killicki* [13] is debated. The species endemic in the Mediterranean are *L. infantum*, *L. tropica*, *L. killicki* and *L. major.*

The species complexes of subgenus *Viannia* are endemic exclusively in the NW:

- *L. braziliensis* complex with the species *L. braziliensis* [Viannia (1911)] and *L. peruviana* [Velez (1913)]
- *L. guyanensis* complex with the species *L. guyanensis* [Floch (1954)], *L. panamensis* [Lainson and Shaw (1972)] and *L. shawi* [Lainson *et al.* (1989)]
- *L. naiffi* complex with *L. naiffi* [Lainson and Shaw (1989)]
- *L. lainsoni* complex with *L. lainsoni* [Silveira *et al.* (1987)]

increased average temperatures could enable extension of sand-fly breeding seasons, or into areas where low temperatures have so far prevented their over-wintering in larval stage. In this context, the main contribution of *Leishmania* genotyping is probably species identification in new endemic settings but it can also establish if the new cases are due to multiple imports or epidemic spread of single isolates. In Europe, *L. infantum* has reportedly spread northward in continental Italy [19]. Typing of MON-1 parasites by PCR-RFLP has confirmed the role of dogs (or canids) as the source of autochthonous human infections in the new areas [20]. In southern Germany, undisputable autochthonous infections in a child and a horse were identified as being caused by *L. infantum* [21,22]. Molecular identification of sand-fly populations is a useful, complementary tool for prediction of vector capacity to disperse and create new foci of leishmaniasis, as a consequence of climate change. To this end, sequence analyses of mitochondrial cytochrome *b*, microsatellites and ribosomal internal-transcribed spacer 2 have been used to differentiate *Phlebotomus perniciosus* populations from Spain, France and Italy [23–25].

Large-scale anthropogenic environmental modifications are occurring in some eastern and southern Mediterranean territories. Population growth, urbanization, introduction of new agricultural practices and civil unrest are largely affecting the West Bank and Israel. Recent reports suggest that *L. tropica* is spreading rapidly in these areas [26]. Genotyping at species level has contributed greatly to monitoring the relative frequency of CL cases due to *L. major* or *L. tropica* in areas where these parasites have become sympatric [27] or to incriminate novel vectors and reservoir hosts [28]. Microsatellite typing of strains of *L. tropica* revealed the emergence of overlapping genetically

Table 2. Comparison of molecular epidemiological tools used for Mediterranean leishmaniasis.

Target	Isoenzymes[a]	Fingerprint	kDNA	cpB/gp63	ITS1	Housekeeping genes	Microsatellites
Method	MLEE	DNA-FP RAPD	PCR-RFLP	PCR-RFLP	Sequencing	MLST sequencing	MLMT fragment analysis
Resolution							
Species	✓	✓	-	✓	✓	✓	-
Clade	✓	✓	-	✓	✓	✓	✓
Strain	-	(✓)	✓	(✓)	(✓)	-	✓
Sample type	Culture	Culture	Clinical specimen	Clinical specimen	Clinical specimen	Clinical specimen	Clinical specimen
Reproducibility	Good	Poor	Poor	Good	Good	Good	Good
Interlaboratory comparison	Possible if same methodology	Difficult	Difficult	✓	✓	✓	✓
Co-dominance	✓	-	-	-	-	✓	✓
Neutrality	✓/-	✓	Not determined	-	Not determined	✓/-	✓
Simple	-	✓	✓	✓	-	-	-
High-throughput	-	-	-	-	✓	✓	✓

Abbreviations: cpB, cystein *protease* B; gp63, major surface glycoprotein; ITS1, ribosomal internal transcribed spacer 1; kDNA, kinetoplast minicircle DNA.
[a]'✓' indicates yes, whereas '-' indicates no. ✓/- means that it will be yes/no depending on the locus tested. (✓) stands for limited discriminatory power for strain differentiation depending on the species investigated.

distinct populations in Israeli and Moroccan foci related to the appearance of new vector(s) and to the possible coexistence of anthroponotic and zoonotic transmission cycles [13].

Strain typing by DNA fingerprinting suggested that the re-emergence of VL in central Israel was not related to a spread south from the old northern focus but to clonal expansion of existing *L. infantum* parasites owing to changing conditions and increased mobility of infected canines in the central region [29]. In Tunisia, where VL transmission was limited to northern humid areas, intensive agriculture projects led to the establishment of a stable cycle of viscerotropic *L. infantum* below the Atlas Mountain chain with a tendency to extend southward [30]. Recently, PCR-RFLP genotyping of CL agents from central Tunisia has uncovered the spreading into this region of dermotropic variants of *L. infantum*, confined previously to the northern part of the country [31]. Analogously, *L. killicki* has been spreading from the original southern Tunisian focus [11]. This underlines the need for parasite genotyping, especially in southern Mediterranean foci where CL can be caused by at least three *Leishmania* species (*L. infantum, L. major* and *L. tropica*), each characterized by distinct epidemiological features. Furthermore, considering the current worldwide mobility, colonization by new species should not be excluded. For example, Latin America was colonized by *L. infantum* after the conquista era and exotic species could invade Mediterranean countries.

Treatment failure and drug resistance

Clinical treatment failure and drug resistance in parasites should be distinguished carefully because they concern different phenomena: a clinical phenotype caused by parasite–host–drug interaction and a feature of the parasite selected through contact with the drug, respectively. Both phenomena were related in Bihar, India [32], but this was not the case in Nepal [33] and Sudan [34].

In the Mediterranean region, there is no major concern currently about treatment failure in humans, apart from HIV-coinfected patients (see later). However, there is no structured trans-border epidemiological surveillance system, so the issue might go undetected. In Italy, a significant decrease in the efficacy of antimonials in humans was observed between 1997 and 2000 [35], leading to the implementation of amphotericin B as the first-line drug. Dogs (reservoir of *L. infantum*) might constitute an important reservoir for the emergence of drug-resistant strains because their treatment is difficult. Therefore, even if some drugs might be registered for humans and dogs, both hosts should, ideally, not be treated with the same drugs, which is a principle that is not always followed.

In this context, molecular epidemiology can be crucial and different applications can be considered. First, species and even strain identification is highly recommended for surveillance of treatment efficacy and for any clinical trials with new drugs. Treatment outcome in patients can vary with *Leishmania* species because of specific differences in drug tolerance [36] and/or interaction with the host immune system. This 'species-effect' is well documented for infections caused by *L. tropica,* which are generally more difficult to treat with antimonials [37]. Second, molecular characterization could be applied to identify drug-resistant parasites. This would be much more efficient than the current *in vitro* susceptibility assays, which are labor-intensive and might not reflect *in vivo* resistance adequately [33,38]. Several candidate genes possibly associated with antimony resistance have been reported [39–41]. The corresponding mechanisms concern mostly differential gene expression, however, and the required quantitative real-time PCR assays need large amounts of amastigotes, making direct application to clinical samples difficult. Surveying resistance to the recently introduced drug miltefosine seems to be easier because genomic markers were found in *in vitro*-induced resistant strains [42]. However, it is unknown if mechanisms would be the same in clinical-resistant strains. Further work is needed to develop molecular tools for the detection of drug-resistant parasites. Third, characterization of the parasites at strain level could demonstrate whether a recurrent episode of leishmaniasis is owing to treatment failure or re-infection. Furthermore, it would help to understand the dynamics of drug resistance in nature. Emergence and spreading of drug resistance might be influenced by several factors,

such as disease-transmission cycle (zoonosis versus anthroponosis), parasite reproduction mechanisms, stability of the resistant phenotype in the absence of the drug and virulence of resistant parasites. These crucial issues could be addressed by parasite-population studies using highly discriminatory markers. In Nepal, the polyclonal structure for antimonial-resistant strains of *L. donovani* suggests independent events lead to the emergence of drug resistance [43]. A similar approach could be performed in the Mediterranean region comparing *L. infantum* populations from treated and untreated dogs with those from humans.

Immunodeficiency and leishmaniasis

Leishmaniasis has been identified as an opportunistic infection in immunosuppressed individuals, especially in those with HIV infection, and less frequently in those who have undergone organ transplant, chemotherapy for malignancy or suffer from immune-mediated disorders. Immunosuppression is one of the factors responsible for increased susceptibility to primary *Leishmania* infection or to reactivation of a silent infection. From 1985 until the late 1990s, *Leishmania*–HIV coinfection was considered to be an emerging disease in southern Europe, where 25–70% of adult cases were related to HIV infection and up to 9% of AIDS cases suffered from newly acquired or reactivated VL [44]. Since the introduction of highly active antiretroviral therapy (HAART), the incidence of VL in HIV^+ patients has dropped in Europe, although relapses still occur [45]. *Leishmania* and HIV coinfections have been reported in 35 out of the 88 countries in which leishmaniasis is endemic [44] and are a growing concern in eastern Africa, Brazil and the Indian subcontinent, where both diseases overlap geographically.

Molecular tools have proven useful for diagnosing VL in HIV coinfected patients because approximately 40% of them do not develop a humoral immune response to the parasite and cannot be diagnosed by serological methods [46]. Furthermore, PCR with subsequent species identification can avoid false-positive results because infections with lower trypanosomatids have been described in immunocompromised patients who displayed symptoms of CL or VL [47].

With increasing international travel, cases of imported leishmaniasis are becoming more frequent [48]. Immunocompromised persons visiting endemic areas might suffer from *de novo* infection with *Leishmania*. MLEE has shown extreme variability of *L. infantum* in subjects coinfected with HIV with the presence of zymodemes, which have never been described in immunocompetent individuals [49,50]. However, more than half of *L. infantum* stocks isolated from HIV patients belong to the MON-1 zymodeme [50]. MLMT and PCR-RFLP of kinetoplast DNA minicircles have the highest discriminatory power available to distinguish strains of *L. infantum* MON-1 from different hosts and endemic areas [51]. Kinetoplast minicircle DNA (kDNA) PCR-RFLP has been applied successfully to: (i) confirm a VL outbreak among HIV^+ intravenous drug users in northeast Spain [52]; (ii) distinguish between relapses and reinfections in coinfected patients [15,17]; (iii) support the existence of separate anthroponotic and zoonotic cycles on the island of Majorca [16] by analyzing *L. infantum* stocks from HIV^+ and immunocompetent patients, dogs and sand flies, and (iv) identify genotypes that are associated with the immune status of the patients in Portugal and confirm the transmission cycles for those genotypes [17].

Molecular techniques have also confirmed that the same strain of *L. (Viannia) braziliensis* was responsible for different clinical manifestations in a kidney-transplant patient [53]. Immunosuppression probably contributed to the dissemination of the parasite. Leishmaniasis can affect transplant recipients owing to recrudescence of a latent infection or *de novo* infection or if they receive a parasitized organ [54]. Molecular markers could help identify the sources of infection in leishmaniasis cases associated with organ transplant or with blood transfusions.

Concluding remarks and future directions

The epidemiology of the leishmaniases is changing not only around the Mediterranean basin but also in other endemic areas in the Old and New World owing to human-made and environmental changes, immunosuppression and increasing unresponsiveness to first-line treatment. Key epidemiological questions require new or improved tools that enable the differentiation of the parasites at species and strain levels. The current gold standard, MLEE, has some drawbacks, such as the need for cultivated parasites and a lack of discriminatory power. It should be replaced by PCR assays identifying species and even strains directly in clinical samples that have proven useful in numerous field studies. Single-target typing, such as PCR-RFLP of ribosomal internal-transcribed spacer [55] is often sufficient for typing at the species or complex level. However, owing to recombination and genome mosaics within species [56,57], more than one target might be required for accurate strain assignment. The first steps have been taken to develop multilocus sequence typing (MLST) [56–58], which is potentially the most powerful phylogenetic approach and will, probably, replace MLEE advantageously in the future. Microsatellite typing and kDNA PCR-RFLP are further able to differentiate below the zymodeme level and, especially microsatellites, have great potential for population genetic studies. Because of its improved reproducibility and possibility of data storage and exchange, MLMT seems to be, currently, the best candidate for becoming the gold standard for strain-level differentiation. However, typing and analytical tools need to be further improved. There is an urgent need in the Mediterranean area for better regional epidemiological surveillance, exchange of information and common analysis of disease trends. Molecular methods make identification and characterization of parasites possible in samples taken directly from the field and representing infected persons, sand flies and reservoirs. Accessible databases should be created and sustained for integrating the data obtained by different researchers. This would enable global analyses and help to avoid biases in analyses owing to small sample sizes.

Molecular epidemiology has great potential for progressing studies on *Leishmania* epidemiology and disease control by enabling the synergistic and productive integration of laboratory scientists and epidemiologists. Unfortu-

nately, this integration is still difficult and many recent studies would have benefited from more interaction between the disciplines. Integration is one of the main challenges ahead.

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