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Species Typing in Dermal Leishmaniasis

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SUMMARY

Leishmania is an infectious protozoan parasite related to African and American trypanosomes. All *Leishmania* species that are pathogenic to humans can cause dermal disease. When one is confronted with cutaneous leishmaniasis, identification of the causative species is relevant in both clinical and epidemiological studies, case management, and control. This review gives an overview of the currently existing and most used assays for species discrimination, with a critical appraisal of the limitations of each technique. The consensus taxonomy for the genus is outlined, including debatable species designations. Finally, a numerical literature analysis is presented that describes which methods are most used in various countries and regions in the world, and for which purposes.

INTRODUCTION

Leishmania species are digenetic parasitic protozoans of the order Kinetoplastida and the family Trypanosomatidae. They are transmitted by phlebotomine sand flies to wild and domestic animals and to humans. Cutaneous leishmaniasis (CL) is one of the main clinical manifestations of human infection (reviewed by Reithinger et al. [1]). In contrast to visceral leishmaniasis (VL), it is not lethal but is often traumatic and associated with social stigmatization. According to a recent estimate (2), the annual incidence of the disease is between 0.7 and 1.2 million cases worldwide. It is endemic in 87 countries, 10 of which (Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Sudan, Costa Rica, and Peru) bear 70 to 75% of the burden (2). All species of *Leishmania* that are pathogenic to humans can cause cutaneous disease, albeit with various severities.

On one side of the spectrum is *Leishmania major*, which generally inflicts self-healing localized skin lesions at the site of the infection (localized CL [LCL]) and is found in the Mediterranean region, Africa, the Middle East, and further, as far as India. On the opposite side one finds *L. braziliensis*, which is endemic in South America and is able to cause severe mutilating mucocutaneous leishmaniasis (MCL) even years after cure of the initial local lesion. Between these extremes are species inflicting difficult-to-treat localized lesions and those often giving rise to diffuse cutaneous leishmaniasis (DCL), whereby several lesions occur distant from the site of infection (1).

To complete the picture, *L. infantum* (syn., *L. chagasi*), found in Mediterranean countries and Brazil, can lead to both CL and VL in humans and to canine leishmaniasis in dogs. VL (also known as kala-azar) is a lethal condition if untreated, with parasites affecting the liver, spleen, and bone marrow. Also, *L. donovani* causes VL, whereby cured patients sometimes develop a particular cutaneous complication known as post-kala-azar dermal leishmaniasis (PKDL), which is not seen with *L. infantum* and manifests as nodules covering large parts of the body (3). Especially in Sri Lanka, *L. donovani* infection often leads to CL (4, 5), making dermal leishmaniasis a disease that can be provoked by all *Leishmania* species that are infectious to humans.

The association between different clinical forms of CL and specific *Leishmania* species is, however, not absolute. The best example is probably mucosal leishmaniasis (ML), which is generally encountered in patients infected with *L. braziliensis* but has also been reported for other species of the *Leishmania* (*Viannia*) subgenus, such as *L. guyanensis* (6). Especially with HIV coinfection and in immunosuppressed individuals, unexpected clinical presentations may be encountered (e.g., see reference 7). Also, the traditional geographical connotation of clinical forms should be taken with caution, because expansion or movement of transmission cycles (e.g., see references 8 to 11) may cause unexpected species to circulate in unexpected regions or habitats.

It is thus clear from the above information, and also because species display specific transmission patterns, affect disease prognosis, and may differentially react to certain drugs or treatment regimens, that efficient control of dermal leishmaniasis requires species typing (e.g., see references 12 to 19). Besides parasite identity, other factors that guide the choice of treatment are the clinical presentation, the host's genetic background and immunity, and additional confounding conditions (1). The need to determine the infecting *Leishmania* species depends largely on the specific context. For clinical and epidemiological studies, confirmation of the infecting species is definitely recommended in all cases (19), preferably using a globally applicable technique that has been validated on all species. In these studies, such an approach is certainly feasible.

For day-to-day clinical management, on the other hand, individual species typing is not always achievable, for either technical, logistic, or financial reasons. The most straightforward situation is presented by a primary health center located in a region of endemicity where only one species circulates. In such cases, accurate diagnosis by genus detection is all that is needed, provided that the epidemiology of the area is sufficiently monitored. Such genus detection can be simple and based on clinical or microscopic examinations of lesions, even though these are not the most sensitive or specific methods. Serology is not very sensitive because of the strong Th1 bias, and molecular methods are superior when the parasite load is low (20). A more complicated setting is that of a primary health center located in a region where two or more species or variants are transmitted, each requiring a different treatment approach. In such a scenario, experienced physicians may

separate species based on the lesion morphology and clinical syndrome. If this is not possible, then the species can be identified using techniques separating the local parasite variants. Furthermore, a regional or national reference center typically deals with patients infected in various environments, which makes individual species identification more relevant, as the exact geographical origin of infection and the local epidemiology are often difficult to assess. Finally, the most complicated setting is that of a travel clinic seeing patients who have visited several regions or countries of endemicity. Generally, the exact region of infection is unknown, and treating physicians have less knowledge of or access to epidemiological information, in addition to often having less experience with diagnosis and treatment. Such an environment hence requires a globally applicable species typing strategy.

This review aims to give a state-of-the-art overview of the currently deployed and available *Leishmania* species typing assays in the context of clinical and epidemiological studies and disease management. The focus lies on methods that are globally applicable rather than on those validated locally. The limitations of each technique are discussed. In addition, current problems with the taxonomic framework of the genus are treated from a practical point of view, and suggestions toward a more solid approach are formulated.

LEISHMANIA TAXONOMY

A Bird's-Eye View

The taxonomy of the *Leishmania* genus is outlined in Fig. 1, essentially as compiled by Schönian et al. (21), but with the addition of *L. siamensis*, recently described from Thailand (22); *L. martiniquensis*, from the French West Indies (23); and *L. adleri* and *L. hoogstraali* (24). This classification of *Leishmania* is by no means undisputed, but neither is any other proposed scheme. The different layers between the classical Linnaean levels of genus and species and the growing number of described species are illustrative of the troublesome classification of the genus (25). Several species have been grouped into so-called "species complexes," further referred to as "complexes," whereby each complex is named after one of the constituting species. Complexes and species were assigned to subgenera, which in turn were grouped into sections or divisions (Fig. 1).

The lack of an unequivocal classification scheme is not unique to *Leishmania* and is essentially the result of using different markers to study phylogeny and of the lack of a universally applicable species definition for unicellular organisms. Multilocus enzyme electrophoresis (MLEE) (26) laid the basis of current *Leishmania* taxonomy, but species were often defined when only minimal differences in isoenzyme characteristics were observed, while these really concerned intraspecies variability or convergent evolution (27). With the advent of molecular techniques to study the variability of genera, it became possible to test the MLEE-derived classifications with more informative genetic methods, and this gave rise to various adaptations. Some of these have now been generally accepted, but others remain subjects of debate (28).

When it comes to identifying species for human or veterinary clinical practice, the situation is not dramatic, however, and almost all authors classify parasites as one of the species underlined in Fig. 1. Generally the species complexes are easily discriminated by many techniques, but often identification of the individual species poses more problems (see "Clinically Relevant Taxa: Certainties and Doubts"). The reason behind this is that species belonging to the same complex are often very closely related, which not only complicates their identification but also hampers an unambiguous species definition. Sometimes more variable markers are needed to distinguish species within the same complex than to distinguish different complexes.

Evaluation of Current Taxonomy

One may ask how species should be defined from a clinical perspective. Some would argue that a species is a set of organisms that impose identical clinical presentations on humans or animals. However, this is not a workable criterion for several reasons. First, what is regarded as clinically identical may change over time. New drugs may become available that specifically affect one group of parasites, while another group shows intrinsic tolerance. Also, vaccines targeting one particular group of parasites may be deployed. Second, clinical presentations may differ according to the geographical region or human population, for instance, in travelers versus individuals with endemic cases (18). Third, for prevention and control, different species can have different transmission cycles, requiring other control measures.

Conversely, some species are clinically pleomorphic. For instance, *L. braziliensis* causes MCL in some patients after cure of a CL lesion. Currently, no difference has been identified between parasites from CL or MCL lesions, and the clinical presentation seems to be governed by the immune system of the host or other confounding factors rather than by the parasite. In such cases, it is impossible to separate isolates into different species.

Consequently, the only logical definition of species relies on parasite identity, which is best studied based on molecular characteristics, such as those revealed by zymodeme and DNA analyses, as these reflect the parasites' evolution. This is also known as the phylogenetic species concept (28) and forms the basis for the current *Leishmania* taxonomy. Inevitably, the next question to address is how different two groups need to be for them to be assigned to different species. It was one of the pioneers of modern evolutionary theory, Charles Darwin, who already observed the difficulty of defining species in higher sexual organisms (29), and it is even much more challenging in single-celled protozoa, such as *Leishmania*. In such cases, defining the species boundary is a matter of convention once the evolutionary relationships have been established, and what may be considered distinct populations in one genus may be regarded as species in another group of organisms.

In this respect, the current *Leishmania* classification is not entirely consistent. As can be observed in Fig. 2, some species are so closely related to each other that one can question their validity as distinct species, even though discriminating them is clinically and epidemiologically relevant. In particular, *L. infantum*, *L. peruviana*, and *L. panamensis* are subgroups of *L. donovani*, *L. braziliensis*, and *L. guyanensis*, respectively, as further detailed in "Clinically Relevant Taxa: Certainties and Doubts."

A further taxonomic complication is presented by the occasional occurrence of natural interspecies hybrids (30–38), although these are quite rare. At the intraspecies or population level, sexual recombination might be frequent (39–44), but in general, a clonal mode of propagation is observed when looking at the greater picture (45). In theory, interspecies hybrids may give rise to new species, which develop their own characteristics by combining those of both parents. This happened for two discrete typ-

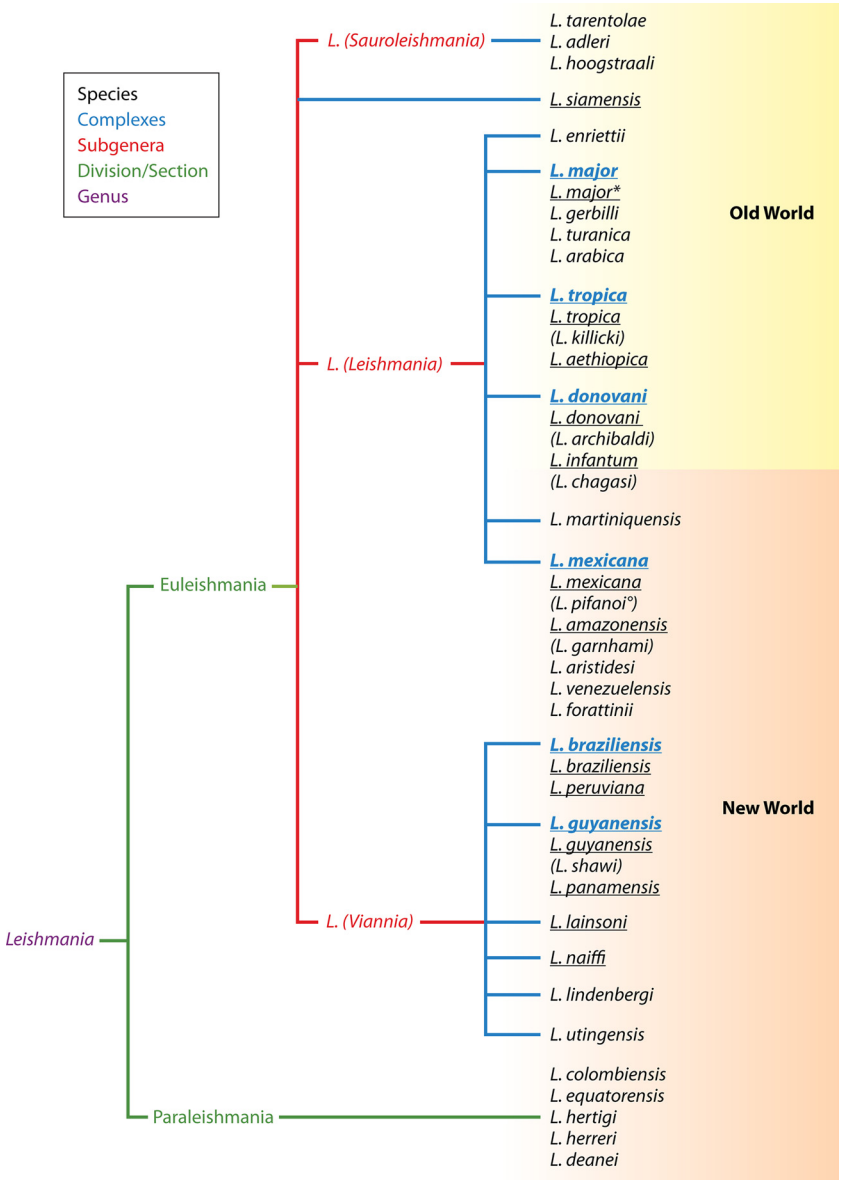

FIG 1 MLEE-based taxonomy of the *Leishmania* genus as listed by Schönian et al. (21), but with the addition of *L. siamensis* (22), *L. martiniquensis* (23), *L. adleri*, and *L. hoogstraali* (24). The various levels are indicated by their respective colors. Several species (black) are grouped into "species complexes," or "complexes" (blue), whereby the complex is named after one of its species. The underlined species are those documented in the studies analyzed in Currently Applied Methods, which are those relevant for human and domestic animal diseases. Species names between brackets are not recognized as separate entities by most authors and in fact are part of the species listed above them. *L. chagasi* is a synonym of *L. infantum* of the New World. °, some *L. pifanoi* strains are more related to *L. amazonensis* than to *L. mexicana* (61, 62); *, several authors have reported *L. major*-like parasites from the New World (62, 79–83). The figure does not represent a dendrogram with evolutionary relationships but a practical classification system.

ing units (DTUs) in *Trypanosoma cruzi* (46), but as far as we know, it has not occurred in *Leishmania*, where interspecies hybrids have been confined in space and time. Nevertheless, they do seem to exist as isolated populations but have not gained any formal taxonomic status. In a specific epidemiological or clinical context, it may, however, be relevant to identify them (see "Detection of Interspecies Hybrids"). Finally, taxonomy is troubled by intermediate forms between closely related parasites, such as those between *L. braziliensis* and *L. peruviana* (33). Some of these may represent remnant transition forms of the speciation process or may represent interspecies hybrids (38, 47). Such forms complicate the clear distinction between species, also leading to difficulties in the species typing process.

Clinically Relevant Taxa: Certainties and Doubts

Several species and species complexes form separate entities that are clearly different from all others. In limiting the list to clinically relevant groups, this applies to *L. siamensis*, *L. major*, the *L. tropica* complex, the *L. donovani* complex, the *L. mexicana* complex, the *L. braziliensis* complex, the *L. guyanensis* complex, *L. lainsoni*, and *L. naiffi* (Fig. 1 and 2). Within the 5 complexes, several typing and species definition problems are observed.

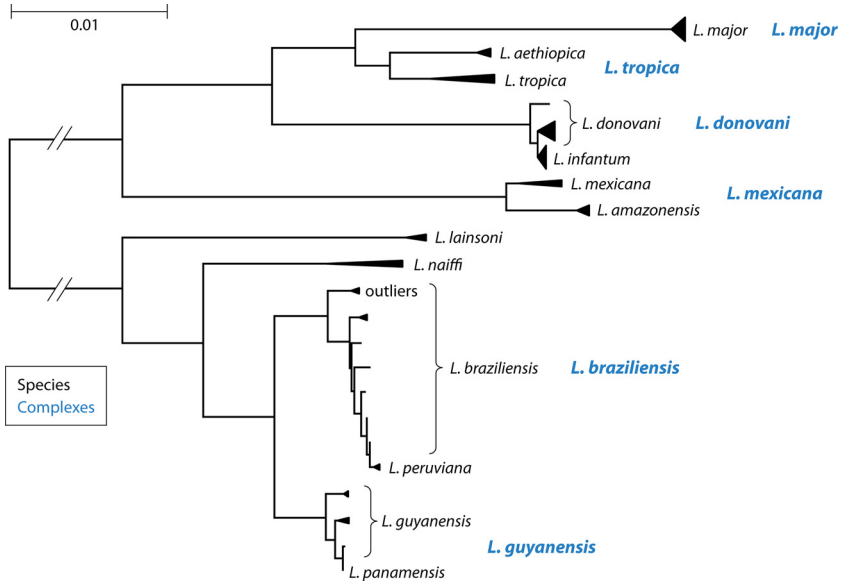

FIG 2 Multilocus sequence analysis of Van der Auwera et al. (60), based on sequences of 7 housekeeping genes. Each species and species complex are indicated, as in Fig. 1. *L. braziliensis* outliers are discussed in "Clinically Relevant Taxa: Certainties and Doubts." *L. shawi* was not included in this analysis, and *L. panamensis* was represented by a single strain. The dissimilarity scale is depicted in the top left corner, in substitutions per nucleotide.

***L. donovani* complex.** *L. infantum* strains are a clear subgroup within the *L. donovani* complex, but other equally valid subgroups identified from microsatellites, amplified fragment length polymorphisms (AFLP), and multilocus sequence typing (MLST) have not been assigned separate species names (48–52). *L. chagasi* was formerly separated from *L. infantum* because of its New World endemicity, but it is now regarded as the same species, which was imported from Europe to Latin America in the 16th century (53, 54). However, Marcili et al. (24) could discriminate them. *L. archibaldi*, which is an MLEE-defined species of the complex (55), was not validated as a monophyletic subgroup by DNA analyses and is no longer used by most authors (27, 49, 51). The species *L. donovani* is thus defined by exclusion and comprises all strains of the complex that are not *L. infantum*. Nevertheless, *L. donovani* strains have some common characteristics, such as their presumed anthroponotic transmission and the PKDL complication. Because both species are close, methods that are fit for typing to the species complex level are often not able to distinguish them.

***L. tropica* complex.** *L. aethiopica* and *L. tropica* belong to the *L. tropica* complex, but these species seem quite well defined genetically, as evidenced by MLST (56). Consequently, most methods can distinguish them. *L. killicki*, on the other hand, behaves more like a local *L. tropica* variant and is better not regarded as a separate species (56–59).

***L. mexicana* complex.** In clinical reports, only two species of the *L. mexicana* complex have been described: *L. amazonensis* and *L. mexicana*. The complex seems to be understudied, because most reports dealing with the *Leishmania* (*Leishmania*) subgenus focus on the Old World, and conversely, most studies of the New World focus on the *Leishmania* (*Viannia*) subgenus. This often leaves the *L. mexicana* complex in an orphaned position, as it is a *Leishmania* (*Leishmania*) subgenus complex of the New World. No comprehensive studies covering the full genetic and geographic variability of the complex are available.

Multilocus sequence analysis identified *L. amazonensis* (including *L. garnhami*) as a clearly defined group in the complex (Fig. 2) (60), which agrees with other markers (61). Also, Schönian et al. (21) considered *L. garnhami* to be a synonym of *L. amazonensis*. *L. pifanoi*, another species of the complex, seems to form a heterogeneous group and is not a valid species. Some strains have been found to be more related to *L. mexicana*, and others to *L. amazonensis* (61, 62).

***L. braziliensis* complex.** *L. peruviana* and *L. braziliensis* have been described as the only species in the *L. braziliensis* complex, based on a minor difference in MLEE profiles (47, 63). If *L. peruviana* is considered a different species, then the situation is comparable to that of the *L. donovani* complex, with *L. braziliensis* being defined by exclusion of *L. peruviana* (Fig. 2). The situation is more complicated, however, as molecular analyses revealed the existence of several intermediate strains that may be hybrids or transitional forms between both species (33, 38, 64).

Current data hence do not support a clear-cut dichotomy between *L. peruviana* and *L. braziliensis*, leading to frequent problems in identifying both species. Nevertheless, in contrast to infection with *L. peruviana*, infection with *L. braziliensis* can result in mucocutaneous complications after the initial cure (65), which is why identification of these species has a pronounced clinical relevance. Currently, full-genome sequences are being determined for several members of the complex, which may help to better define both species (unpublished results).

Some studies identified a cluster of *L. braziliensis* strains that are genetically distinct from the main *L. braziliensis*-*L. peruviana* group ("outliers" in Fig. 2), comprising parasites from different countries (33, 60). This group has been given various names, such as "outlier *L. braziliensis*" (66), "atypical *L. braziliensis*" (67), and "*L. braziliensis* type 2" (60). It has so far not been widely recognized, even though it is discriminated much more easily than *L. peruviana*. Most assays probably type the group as *L. braziliensis*. Other authors reported an atypical *L. braziliensis* group on the basis of MLEE and DNA sequence analysis (68), but it is unclear

whether this concerns the same variant. A more detailed phylogenetic study is needed to decide the taxonomic status of the *L. braziliensis* outliers, as some markers place them at the same distance from *L. braziliensis* as other species complexes (33).

***L. guyanensis* complex.** The *L. guyanensis* complex unites the species *L. guyanensis*, *L. panamensis*, and *L. shawi* (Fig. 1). Members of the complex are widespread in Latin America and have been reported from Brazil, Venezuela, Colombia, Peru, Ecuador, Surinam, French Guiana, Guyana, Argentina, and Central American countries, such as Panama and Costa Rica. So far, studies have been limited to analyzing strains from a particular geographical origin or country, and the entire genetic variability of the complex has not been assessed. Nevertheless, even based on a limited number of strains, several reports have questioned the species status of *L. panamensis* (69) and *L. shawi* (41). Given the current evidence, it can be extrapolated that *L. panamensis* and *L. shawi* may be local variants of *L. guyanensis*, which would then be defined by exclusion of the two subgroups (Fig. 2).

Looking for Answers and Finding Pragmatic Solutions

A common denominator underlies all the above-mentioned problems with current taxonomy, namely, the lack of comprehensive studies that include the entire variability observed in each complex (28). Analyzing a globally representative set of strains is the only possibility for determining the exact status of each entity as either a complex, species, or subspecies. Adequate sampling not only relates to geographical coverage but also to including strains from asymptomatically infected individuals, animal reservoirs, and vectors; otherwise, one risks looking just at the tip of the iceberg. However, for clinical practice, it may be more convenient to discriminate disease-causing parasites, i.e., those isolated from actual lesions, even though this is not recommended for establishing a general taxonomy of the genus.

After gathering all samples, the next question is how to analyze them. Studying the population structure of each complex seems to be the way to go in order to define well-characterized groups, but on which data should this be based? MLEE has the best coverage of genus variation, at least for cultured strains, and remains advocated as the gold standard by the World Health Organization (65). Unfortunately, this method is not able to characterize uncultured parasites and is based on a limited number of mutations affecting physicochemical enzyme properties. The advent of next-generation sequencing might be taken as an opportunity to exploit the entire genome (70), certainly as new developments are in the pipeline that may allow whole-genome sequencing even from clinical samples (71). Taking into account the vast information content from a full-genome sequence compared to that obtained from MLEE, this would be the method of choice. Multilocus sequence analysis is the next best option, as it uses sequence information from various genes distributed over different chromosomes (72).

Taken together, the data indicate that characterizing a global panel with the best techniques available is the ideal scenario in the quest for defining a useful and unbiased reference framework for the classification of *Leishmania*. This requires a global effort, however, which is not likely to happen in the near future, for financial or political rather than technical reasons. A profound study of each species complex is more realistic, but even that requires extensive additional sampling. At present, studies documenting parasite diversity in-depth focus on particular local issues only (e.g., see reference 73).

For both human and veterinary clinical practice, the species underlined in Fig. 1 provide an adequate framework despite the open questions for some species complexes. The following sections present an overview of how these species can be identified by using the methods at hand in whatever setting one works in. These methods are a practical approach allowing one to conduct clinical and epidemiological studies and also enabling optimization of patient care on a day-to-day basis.

METHODS FOR SPECIES TYPING

Levels of Typing and Focus of This Review

The current review primarily discusses typing methods that have the ability to discriminate many different species or complexes in a clinical context. Each technique looking at parasite variability is designed for a particular purpose, and three steps can be discriminated in a diagnostic workflow.

The first stage usually targets detection of the *Leishmania* genus rather than a specific species (20, 74–77). Assays focusing on this level are not covered here, except when species typing relies on downstream analysis of PCR amplification products generated in the detection process. Methods designed for detection of the genus should be sensitive and *Leishmania* specific.

After *Leishmania* infection has been confirmed, the subsequent step involves identification below the genus level, down to the subgenus, species complex, or species level. In contrast to the *Leishmania* detection tools described above, the most useful typing assays were designed to discriminate many different species, with less emphasis on sensitivity. These tests are the subject of this paper. Unless otherwise specified, we focus specifically on discriminating clinically relevant species, i.e., those underlined in Fig. 1, without relying on parasite isolation in culture. Methods applicable to clinical samples are prioritized over those used for sand flies, as insects can harbor additional species and genera that are not encountered in humans. We highlight *Leishmania* species typing methods that are applicable for everyday use, discriminate many species, and have been validated in a large geographical area.

In particular cases, i.e., population or epidemiological studies, it may be relevant to discriminate isolates to below the species level, or even to the strain level. This involves methods with higher discriminative potential, which are outside the scope of this review unless such methods are also useful for species typing.

The distinction between these three diagnostic levels is not always clear-cut. In some settings, the genus detection step is replaced by a sensitive assay that targets a particular complex, species, or subgenus rather than the entire genus. Such methods are able to identify a particular group of *Leishmania* parasites, but they are useful only when no other variants of the parasite are expected. This can be difficult to establish in the current context of human mobility, urbanization, and changing climate and ecology. On the other hand, some species typing methods also allow characterization below the species level.

Criteria for Species Typing Tools

A multitude of different methods have been designed for discrimination of *Leishmania* species, a search that has been going on for many decades, as evidenced in early publications (e.g., see reference 78). They can roughly be categorized by function of the technology involved or by function of the biological feature evaluated.

Regardless, in our opinion, an ideal typing tool for clinical purposes should adhere to the criteria listed here.

First, a species typing tool must be able to discriminate species. But which species should it discriminate? The absolute minimal requirement is that it can identify species circulating in the studied area. In order to assess this, one needs to take into account the entire *Leishmania* variability encountered in the region where the test is to be applied, both between and within species. In many studies describing a new technique or marker, however, validation is performed on only a few reference type strains, often from a region or country that is different from that where the test is used. Given the tremendous variability of single-cell parasites, this is a major flaw, and tests not validated with adequate reference strains must not be regarded as reliable.

Second, the tool is preferably globally applicable. Especially in times of climate change and rapidly changing ecology, evaluating whether a test can discriminate species in a particular area entails a certain risk. It assumes that we accurately know the epidemiology of the area and ignores the possibility of other species invading new territories. For example, a test designed to discriminate *L. major* from *L. tropica* in Kenya may mistakenly identify an *L. aethiopica* sample as *L. tropica* if the test does not distinguish these species. Because up-to-date epidemiological data on a particular study area are often scanty, the safest methods are those validated on a global scale, taking into account *Leishmania*'s worldwide variability. The larger the region where the test can be applied, the better the result will be. For instance, a global test is better than one for the New World, which in turn outperforms one for Colombia. And even in large areas, such as the entire New World, unexpected parasite variants may turn up, such as occasionally documented *L. major*-like parasites, which are expected to be found exclusively in the Old World (62, 79–83).

Third, the assay must be sensitive. For typing of cultured parasite isolates, sensitivity is not an issue, as plenty of starting material is available for processing. However, clinical and epidemiological studies often require culture-independent and high-throughput applications, which must be able to analyze a minimal amount of parasites, even more so when asymptomatic infections are studied. Also, in the diagnostic pipeline, parasite isolation is often replaced by fast and sensitive methods that are directly applicable to clinical samples.

Fourth, the test must be *Leishmania* specific. *Leishmania* parasites are typically present in a high background of human or animal cells, sometimes infected by other organisms. Any cross-reaction with non-*Leishmania* biomolecules must not prevent parasite identification. Therefore, typing methods need to be targeted to *Leishmania*, or at least must be able to discriminate *Leishmania* from other pathogens in the sample. This is a major issue with HIV patients, in whom several trypanosomatids can be encountered (84) but also other coinfections have been documented, such as mycobacteria and *Schistosoma* (28). These problems are less of an issue for analyzing cultured isolates, where *Leishmania* promastigotes form most of the harvested biomass. In cultures, however, other problems may arise, such as other pathogens from the sample outgrowing *Leishmania* (85).

Fifth, standardization is an issue. In order to compare species typing results across different studies, the technology employed should be reproducible in various settings. Some methods are easier to standardize than others. PCR followed by amplicon sequencing, for instance, is quite easily standardized, as (good-quality) sequences leave little room for interpretation. Restriction fragment length polymorphism (RFLP) analysis is already more difficult, as small fragment size differences and incomplete digests may interfere with a correct reading of the patterns. Even more problematic are species-specific PCRs, hybridization assays, and melting assays, where even small experimental changes to the reaction conditions (buffer, pH, ionic strength, thermocycling parameters, optical detection, and amount of sample or parasite DNA) can result in an altered specificity. Currently, no commercial standard tests are available for *Leishmania* species typing.

Sixth, a given tool must be applicable in the setting where it is needed. This is probably what hampers most development of a generic typing technology. What springs to mind when talking of feasibility are limited-resource labs, which often have no access to common technology available in more advanced settings, for example, resources for DNA extraction and PCR. But equally so, large-scale clinical and epidemiological studies may benefit from high-throughput applications not generally of use in a patient management setting, where speed may be a key requirement. Nevertheless, different technologies can be based on the most informative biological information available for species discrimination, such as a given gene target.

Seventh, any assay needs proper validation. Before venturing into species typing for a particular purpose and context, one has to prove that the assay can, with a certain degree of confidence, assign the correct species. This context can be defined geographically but is equally dependent on the kind of sample involved, e.g., samples from humans, animal reservoirs, or vectors, where different *Leishmania* and non-*Leishmania* parasite species may be encountered. Validation is a critical point in any assay development and implementation but is often overlooked, especially for in-house assays. It requires testing the technology with all parasite variants that are circulating, both *Leishmania* and non-*Leishmania* parasites. Participation in an external quality control program can be beneficial in this regard. Validation can be highly simplified by selecting a standardized test that has already been evaluated extensively in various settings. In this respect, using a well-established assay is generally preferred over use of an in-house test.

As illustrated by various examples below, only a minority of currently available species discrimination assays comply with these, in our view, logical test requirements.

Species Typing Assays

Many methods have been described for species typing of *Leishmania* in clinical, environmental, or cultured samples. The following sections give an overview of the different methods and targets currently available, with an emphasis on assays applicable to clinical samples in a large geographical area and on those that are widely used. Some sections deal with a particular method, and others report on the biological target, whichever is more convenient for clarity. Undoubtedly, the list is not exhaustive, and other locally deployed methods can be found in the literature. Nevertheless, the techniques that best abide by the criteria listed above are included. For each method, an appreciation is given of the pros and cons of the technique, and Table 1 presents a comparative overview of the best-validated and most used tests.

MLEE

MLEE is still considered by many (including the World Health Organization [65]) to be the gold standard in parasite typing, even

TABLE 1 Overview of the most used and best-validated typing assays[a,b]

Technique [1]	Reference	Groups [2]	Tissue [3]	*L. (Leishmania)*							*L. (Viannia)*							
				Old World					New World									sia
				aet	tro	maj	don	inf	mex	ama	lai	nai	bra-O [4]	bra	per	guy	pan	
Monoclonal antibodies	98	6	No					4 / 2	3 / 3	4 / 3	1 / 1	1 / 1	7 / 3			3 / 1	4 / 4	
ITS1 RFLP	112	8	Yes	1 / 1	1 / 1	1 / 1	1 / 1	2 / 2 [5]	1 / 1	1 / 1			1 / 1			1 / 1	1 / 1	
ITS1 sequencing	60	11	Yes	3 / 2	4 / 3	11 / 10	10 / 7	10 / 7	3 / 3	5 / 5	3 / 2	3 / 2	2 / 1	7 / 4	3 / 1	5 / 3	1 / 1	
ITS1 reverse line blot	106	5	Yes	2 / 1	2 / 1	5 / 3	6 / 4	5 / 3								1 / 1		
ITS1 specific PCRs	115	4	Yes	10 / 2	12 / 5	11 / 9	8 / 4	8 / 5										
ITS1 melting analysis	102	4	Yes	7 / -	86 / -	52 / -	12 / -	143 / -										
SSU-rDNA + ITS1 RFLP	117	5	Yes					5 / 4	2 / 2	5 / 3	3 / 1	3 / 2	5 / 4		1 / 1	4 / 1		
ITS1 + ITS2 RFLP	118	5	No								1 / 1	10 / 1	17 / 7			9 / 1	4 / 3	
ITS2 sequencing	111	10	Yes	3 / 1	4 / 1	4 / 2	4 / 2	5 / 2	4 / 1	4 / 1			4 / 1			4 / 1	4 / 1	
kDNA minicircle size	134	2	Yes		2 / 2	2 / 1		2 / 2	1 / 1	1 / 1						1 / 1	1 / 1	
kDNA minicircle size	135	2	Yes	1 / 1	1 / 1	1 / 1	1 / 1	1 / 1	1 / 1	1 / 1			1 / 1					
kDNA minicircle size	136	3	Yes	2 / 1	4 / 4	3 / 1	3 / 3	4 / 3	1 / 1	1 / 1			1 / 1			1 / 1	1 / 1	
kDNA minicircle size	137,138	4	No		1 / 1	3 / 2	1 / 1	1 / 1	1 / 1	3 / 1			1 / 1		1 / 1	1 / 1	1 / 1	
kDNA minicircle hybridization	139	4	No		1 / 1	1 / 1	1 / 1	2 / 1	1 / 1	3 / 2	1 / 1		11 / 4		1 / 1	2 / 2	1 / 1	
CytB sequencing	Fig. 7B	12	Yes	1 / 1	3 / 2	2 / 2	3 / 3	3 / 3	3 / 2	4 / 2	1 / 1	1 / 1	4 / 2		4 / 1	3 / 2	1 / 1	3 / 1
gp63 RFLP	170	8	No			1 / 1		1 / 1	1 / 1	2 / 1	2 / 1		35 / 3		17 / 1	4 / 3		
gp63 RFLP	169	5	Yes							1 / 1	4 / 2		16 / 2		16 / 1	7 / 2		
gp63 RFLP	171	5	No	1 / 1	1 / 1	1 / 1	8 / 4	27 / 6										
cpb RFLP	178	4	Yes	1 / 1		1 / 1	5 / 3	10 / 4										
cpb RFLP	184	3	Yes										20 / 2		9 / 1	10 / 3	2 / 1	
hsp70 RFLP	67,196	14	Yes	8 / 2	9 / 5	7 / 7	12 / 4	21 / 8	2 / 2	5 / 3	10 / 2	4 / 2	3 / 2	19 / 3	12 / 1	10 / 4	2 / 1	
hsp70 sequencing	66	15	Yes	8 / 2	6 / 5	15 / 11	16 / 9	17 / 10	4 / 4	6 / 5	4 / 2	4 / 2	5 / 2	19 / 4	6 / 1	8 / 4	8 / 2	4 / 1
hsp70 UTR sequencing	204	8	No	3 / 1	2 / 2	3 / 2	4 / 3	4 / 2		2 / 1	1 / 1		2 / 2		1 / 1	1 / 1	1 / 1	
Mini-exon RFLP	209	10	Yes	1 / 1	2 /2	1 / 1	6 / 5	2 / 2	1 / 1	1 / 1		1 / 1	1 / 1		1 / 1	2 / 1	1 / 1	
Mini-exon sequencing	60	11	Yes	3 / 2	4 / 3	11 / 10	6 / 5	4 / 4	3 / 3	5 / 5		3 / 2	2 / 1	6 / 4	3 / 1	6 / 3	1 / 1	
7SL sequencing	60	7	Yes	4 / 2	3 / 3	11 / 10	10 / 7	10 / 7	3 / 3	5 / 5	3 / 2	3 / 2	2 / 1	7 / 4	3 / 1	6 / 3	1 / 1	
7SL sequencing	211	9	Yes	3 / 1	2 / 2	2 / 2	1 / 1	3 / 2	1 / 1	2 / 2			4 / 2			3 / 1	4 / 3	
7SL melting analysis	103	3	Yes		43 / 15	19 / 8	15 / 6											
G6PD PCR	216	3	Yes						1 / 1	1 / 1	1 / 1	3 / 1	11 / 1			4 / 1	1 / 1	
GPI real-time PCR [6]	219	4	Yes		3 / -	5 / -	3 / -	1 / -	6 / -				2 / -			1 / -	5 / -	
Sequencing 4 genes	68	5	Yes								6 / 1		5 / 1 [7]	14 / 2	14 / 1	8 / 2	10 / 2	
Repetitive DNA	220	5	Yes	2 / 2	11 / 8	5 / 2	4 / 3	10 / 3										

[a] For each assay, groups of species that could be discriminated are indicated by different color patterns, and those not shaded could not be identified. The table is indicative, as each assay was tested on a different set of strains. Species abbreviations: aet, *L. aethiopica*; tro, *L. tropica* (includes *L. killicki*); maj, *L. major*; don, *L. donovani* (includes *L. archibaldi*); inf, *L. infantum* (includes *L. chagasi*); mex, *L. mexicana* (excludes *L. pifanoi*); ama, *L. amazonensis* (includes *L. garnhami*, excludes *L. pifanoi*); lai, *L. lainsoni*; nai, *L. naiffi*; bra-O, *L. braziliensis* outliers (Fig. 2); bra, *L. braziliensis*; per, *L. peruviana*; guy, *L. guyanensis* (includes *L. shawi*); pan, *L. panamensis*; sia, *L. siamensis*. For each assay and species, the first number indicates the number of type strains tested, and the second number gives the number of different countries from which these originated, giving an indication of the intraspecies variability that was evaluated. The number of countries of origin may sometimes be slightly higher, as it was not always mentioned for each strain in the publications.

[b] [1], Techniques are listed in the order that they appear in the text. [2], Number of species or groups of species that could be discriminated. [3], Whether the method, to our knowledge, was used on sand fly or clinical samples. Application in such samples was not necessarily done in the referenced paper and refers to application of the PCR assay rather than the downstream PCR analysis. [4], Most studies did not recognize *L. braziliensis* outliers (Fig. 2) as a separate entity. In such cases, the columns were taken together to represent all *L. braziliensis* strains as one category. [5], *In silico* analysis showed that *L. infantum* could not be distinguished as a separate group when intraspecies variability was taken into account (see "rDNA array" in the text). [6], Countries where the reference strains originated were not mentioned in the referenced paper. [7], Tsukayama et al. (68) described atypical *L. braziliensis* strains, but these might be different from the *L. braziliensis* outliers shown in Fig. 2.

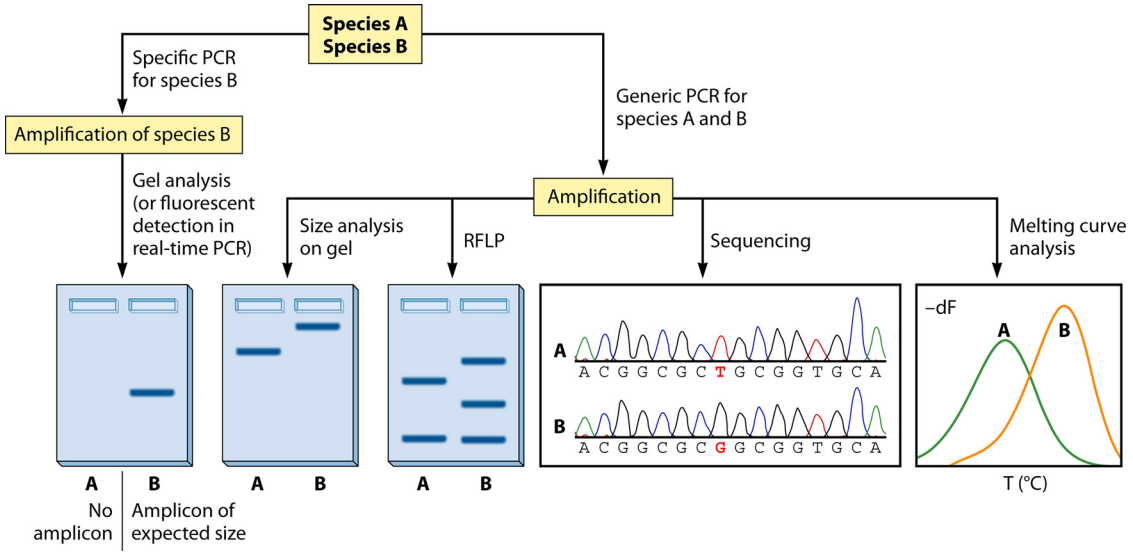

FIG 3 Schematic overview of common techniques to discriminate between two species (A and B). See "PCR-Based Methods" for details.

though the method is cumbersome, time-consuming, only applicable to cultured parasites, and exclusively applied in a few labs across the globe. Because it has been the reference test for so long, it is the only technique that has been evaluated for almost all currently identified *Leishmania* species.

MLEE entails a biochemical characterization based on the pH-dependent electrophoretic mobility of a predefined set of proteins (usually around 10 to 15) in a gel. The combined pattern of all these proteins constitutes a so-called zymodeme, which serves as the basis for species assignment as well as for classification below the species level (30, 55, 58, 86–88). Nevertheless, different labs make use of other enzymes, leading to several identification systems, such as the MON (Montpellier, France) (26), LON (London, United Kingdom) (89), and IOC (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) (90) classifications. As only a few labs nowadays persist in performing these analyses, and the method relies on parasite isolation and culture, MLEE is not suitable as a typing method for everyday use (91).

Other Culture-Dependent Methods

Several authors have described the use of monoclonal antibodies to identify *Leishmania* species of the *Leishmania* (*Viannia*) (92–95) or *Leishmania* (*Leishmania*) (96, 97) subgenus, or both (62, 98–100). These antibodies to some extent specifically recognize cultured promastigotes of different species, or they are genus specific. As such, all assays require parasite isolation and are unfit for analyzing clinical or environmental samples. Grimaldi and McMahon-Pratt (98) described the most extensive validation on nearly all New World species, of both the *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) subgenera, whereby most species could be discriminated (Table 1). No extensive antibody panel has been designed for identifying Old World species. At present, antibodies are rarely used, as more reliable PCR techniques have become available.

Recently, mass spectrometry was suggested for typing of *Leishmania* parasites (23, 101), providing a promising means of rapid identification to the species level. Nevertheless, it is suitable only for identifying cultured parasites, and spectral data are not comparable across different laboratories. Therefore, each center must develop its own validated library of reference spectra.

PCR-Based Methods

The vast majority of methods for species typing, in either a clinical study or an epidemiological, environmental, experimental, or patient management context, involve the use of PCR. Because PCR allows a massive specific amplification of *Leishmania* DNA, it is applicable directly on clinical samples, without the need for parasite isolation. The technique uses either a generic PCR that amplifies any *Leishmania* species or a specific PCR that amplifies a single or multiple species, species complexes, or subgenera (Fig. 3). In using a specific PCR, detection of the PCR product can be achieved either on a conventional agarose gel or in a real-time PCR format using fluorescence detection. In case of a generic PCR, downstream analysis is required in order to determine the parasite species on the basis of size or sequence information in the PCR amplicon.

The most widely accessible and most used sequence-dependent technique is RFLP analysis, whereby the PCR product is digested with one or several restriction endonucleases. Depending on the presence or absence of the enzyme's recognition site, differently sized DNA fragments are generated. Gel-based analysis of the resulting DNA fragment mixture subsequently allows classification of the parasite at hand (Fig. 3). As RFLP is a simple technique that requires minimal lab infrastructure, it is available in each lab where PCR can be done. A more informative and equally straightforward method is sequence analysis of the PCR amplicon. By identification of single-nucleotide polymorphisms (SNPs) or comparison of the obtained sequence with available reference sequences in a dendrogram, such as in Fig. 2, the species can be determined. It should be noted that dendrograms are also constructed for studying evolution, but these analyses require mathematical models that may differ from those applied for typing.

Other techniques have been described but have rarely been used for everyday applications outside the lab where they were developed. Such techniques entail the use of probe hybridization and melting curve assays, in various formats. For example, some au-

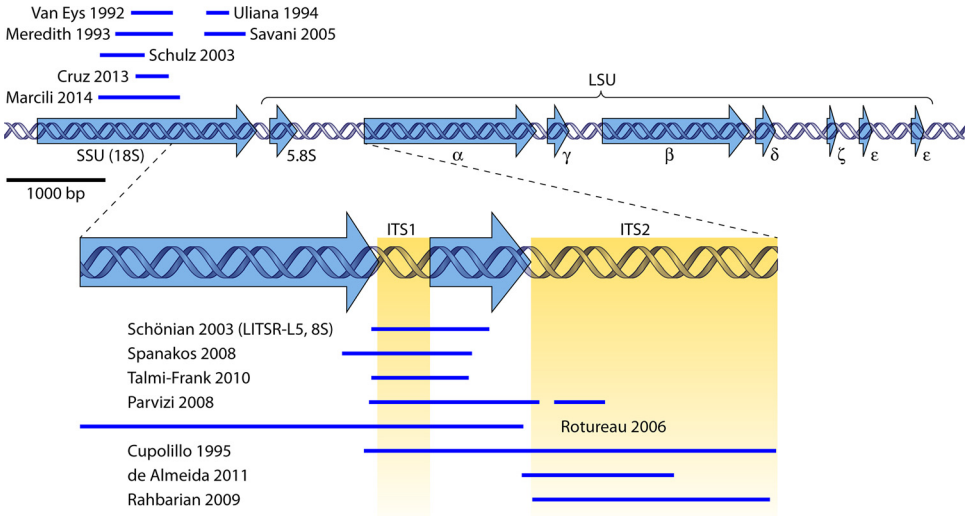

FIG 4 rRNA gene array of *L. major* strain MHOM/IL/81/Friedlin. The sequence and annotations were taken from www.tritrypdb.org (LmjF chromosome 27, nucleotides 989640 to 998595; accessed on 10 August 2014). Arrows in blue indicate the 5′-to-3′ direction of the genes. The lower panel represents a more detailed view of the most relevant fragment for species typing. Regions used by different authors are indicated above (top) or below (bottom) each panel (24, 77, 102, 111, 112, 114, 116–118, 120–124, 126). The scale of the upper panel is given on the left. SSU, small ribosomal subunit rRNA gene (18S rRNA); LSU, large ribosomal subunit rRNA gene; ITS, internal transcribed spacer.

thors have described species-specific melting points of double-stranded DNA fragments or bound oligonucleotide probes (Fig. 3) (102–105). Nasereddin et al. (106) used a reverse line blot assay on a conventional membrane, whereby the similarity between the PCR amplicon and species-specific oligonucleotides was assessed.

The following sections deal with various targets that have been used in PCR-based technology. Some of the enzyme-encoding loci had previously been included in MLEE analysis, and DNA sequence-based approaches were initially developed to study the mutations underlying different zymodemes.

rDNA Array

As detailed in Currently Applied Methods, below, the ribosomal DNA (rDNA) array is one of the most popular species typing targets. In *Leishmania*, an estimated 10 to 20 copies are tandemly repeated per haploid genome, rendering sufficient sensitivity for analyzing clinical sample DNA. Each repeat unit consists of several genes and spacers (Fig. 4) (107, 108).

Within the array, primarily the spacer regions contain sufficient variability for species discrimination, even though they have also been used to discriminate isolates at the subspecies level (57, 109, 110). Some minor sequence variation between the different copies has been observed, which occasionally complicates interpretation of sequence reads (60, 111). It is not clear, however, whether these differences are biological or caused by enzymes in PCR or sequencing reaction mixtures. The following paragraphs give an overview of the fragments and techniques that have been described (Fig. 4).

(i) LITSR-L5,8S. The primers for the LITSR-L5,8S assay were originally reported by el Tai et al. (109). They were applied for species discrimination by RFLP analysis with the enzyme HaeIII by Schönian et al. (112), who evaluated the technique in clinical samples. It allowed separation of species from the *Leishmania* (*Leishmania*) subgenus but did not discriminate *Leishmania* (*Viannia*) species (77, 113). The authors evaluated their method using only one type strain of each investigated species (Table 1), thereby overlooking possible intraspecies variation that could lead to erroneous results.

The amplified fragment was also used for identification by sequencing, allowing a better assessment of the discrimination power obtained with this target. The most extensive analysis was recently carried out by Van der Auwera et al. (60), who evaluated the sequences of a globally representative panel of all medically relevant species (Table 1). Their findings corroborated the RFLP analysis in that the complete sequences could also best discriminate the *Leishmania* (*Leishmania*) subgenus. The sequence analysis included the additional species *L. naiffi*, *L. lainsoni*, and *L. braziliensis* outliers (Fig. 2), also allowing their identification (Table 1).

As sequence variability was observed in nearly all distinguished groups, the question of whether the observed sequence variation hampers correct identification by RFLP analysis using HaeIII remained. To assess this, we undertook an *in silico* RFLP analysis of the data set of Van der Auwera et al. (60), and the results are depicted in Fig. 5. This largely confirmed the previously published RFLP data (112), even though the separation of *L. donovani* and *L. infantum* was no longer possible. This was also observed by Cruz et al. (77) and is caused by the higher level of coverage of intraspecies variation.

Tojal da Silva et al. (30) proposed using Sau3AI for RFLP analysis of this target to allow separation of species of the *Leishmania* (*Viannia*) subgenus. Nevertheless, *in silico* analysis of the global data set of Van der Auwera et al. (60) disproved the possibility of identifying the individual species or species complexes (Fig. 5). Spanakos et al. (114) used an alternative fragment also covering the ITS1 region (Fig. 4), but with ApoI as the restriction endonuclease. Figure 5 includes an *in silico* analysis of the LITSR-L5,8S fragment digested with ApoI, which was less efficient than HaeIII and Sau3AI at discriminating *Leishmania* (*Leishmania*) species.

Besides sequencing and RFLP analysis, a reverse line blot hybridization assay was developed based on the LITSR-L5,8S frag-

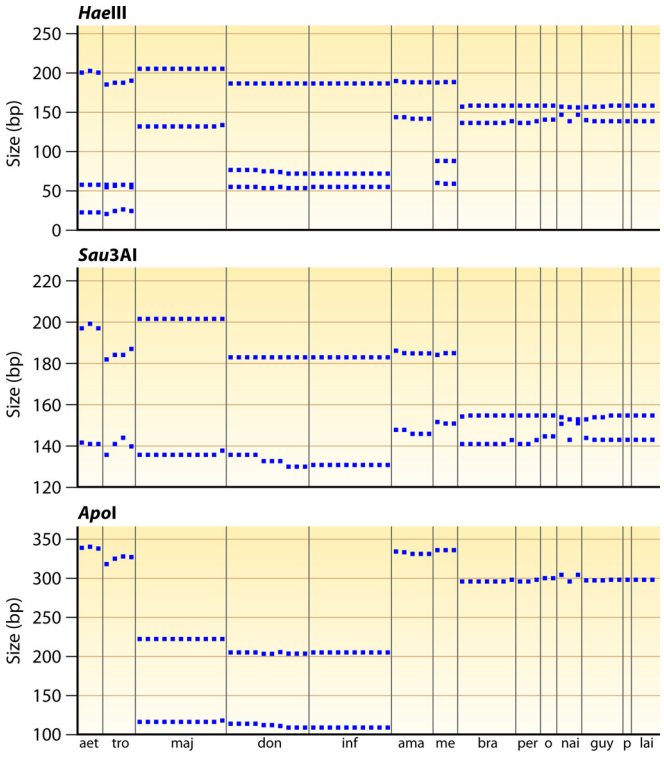

FIG 5 *In silico* RFLP analysis of the ITS1 sequences of Van der Auwera et al. (60), covering the LITSR-L5,8S fragment used by Schönian et al. (Fig. 4) (112). For each of the 3 enzymes, the expected fragments are plotted as a function of size in base pairs, whereby each data point on the abscissa represents a different strain. Species abbreviations: aet, *L. aethiopica*; tro, *L. tropica*; maj, *L. major*; don, *L. donovani*; inf, *L. infantum*; ama, *L. amazonensis*; me, *L. mexicana*; bra, *L. braziliensis*; per, *L. peruviana*; o, *L. braziliensis* outliers (Fig. 2); nai, *L. naiffi*; guy, *L. guyanensis*; p, *L. panamensis*; lai, *L. lainsoni*.

ment (106). In this setup, genus- and species-specific probes are immobilized on a membrane, and reactivity of each of these probes against the PCR product is scored visually. This assay was evaluated on Old World *Leishmania* parasites by use of a few type strains of each species and was able to discriminate all five species (Table 1). It was also evaluated on *L. tropica* and *L. major* samples from patients in Israel, where a perfect correlation with RFLP analysis was demonstrated. Even though hybridization experiments in general are difficult to standardize, the assay uses the probes for all species simultaneously, and therefore a relative measure of hybridization to the various species-specific probes is obtained for each sample, rendering the assay quite robust. In addition, the sensitivity was significantly higher than that of PCR, which is explained by the additional colorimetric signal amplification. With the genus-specific probes, some nonspecific results were observed, but this did not interfere with correct species typing. Despite its promising characteristics, the method has, to our knowledge, only been used in the lab where it was developed.

(ii) Species-specific PCRs. Odiwuor et al. (115) developed a set of species-specific PCRs to discriminate the Old World *Leishmania* complexes. These PCRs are nested within the LITSR-L5,8S fragment described above, which opens possibilities for nested PCR to increase the sensitivity. As Odiwuor et al. pointed out, the 4 species-specific PCRs are best used in parallel to avoid misclassification due to cross-reactions, which would be apparent if a sample is positive for more than one species at a time. This strategy was also used in the above-mentioned hybridization assay (106). Species-specific PCRs need proper validation due to their dependence on minor technical variations, but the authors in this case showed that the technique is transferable to another lab with minimal effort. The assays were validated on various reference strains from each species and from different geographic origins and were able to identify three species and the *L. donovani* complex (Table 1). As for the hybridization assays, these species-specific PCRs were also used only in the labs where they were originally developed.

(iii) High-resolution melting analysis. Talmi-Frank et al. (102) developed a melting assay based on a 265- to 288-bp fragment within ITS1 (Fig. 4). After PCR, the temperature at which both DNA strands separate is recorded with high resolution, and this was found to be species specific. As with RFLP analysis, the Old World species could all be distinguished, with the exception of *L. infantum* from *L. donovani*. The assay was tested on a large panel of isolates from different geographic origins (Table 1) but so far has not been used outside the lab where it was optimized.

(iv) Other ITS1 typing methods. Spanakos et al. (114) described the use of sequencing, RFLP, and single-strand conformation polymorphisms for species discrimination in the Old World, based on an ITS1 fragment that largely overlaps the LITSR-L5,8S target (Fig. 4). These methods showed the same species discrimination as that obtained with the LITSR-L5,8S region but were not as extensively validated. Other examples based on ITS1 sequencing include the work of Parvizi and Ready (116), who used a PCR region covering the LITSR-L5,8S target. Their fragment extended into the 5.8S rRNA gene (Fig. 4), but this did not show any advantage. Rotureau et al. (117) further extended the analyzed fragment into the small-subunit (SSU) rRNA to achieve typing in the New World by RFLP analysis (Fig. 4), but this strategy also failed to discriminate all species, or even all complexes (Table 1).

(v) ITS2 typing. In contrast to the above-described ITS1-based methods, Cupolillo et al. (118) could discriminate all tested species of the *Leishmania* (*Viannia*) subgenus by PCR-RFLP analysis of a fragment covering both ITS1 and ITS2 (Fig. 4; Table 1). However, 10 enzymes were needed, while sequencing of this fragment was found to be complicated because of microsatellite regions in ITS2 (116). Davila and Momen (119) separated most *Leishmania* (*Leishmania*) species on the basis of ITS1-5.8S-ITS2 rDNA sequences, but this showed no added value compared to the use of the LITSR-L5,8S fragment.

Some authors have used exclusively the ITS2 region for typing purposes (Fig. 4). A quite recently published ITS2-based assay was performed by de Almeida et al. (111). They compared typing results from single-nucleotide polymorphism analysis and MLEE, which proved largely congruent, and they could separate the three tested *Leishmania* (*Viannia*) species (Table 1). Other authors (116, 120) identified Old World species based on the size or sequence of various ITS2 fragments (Fig. 4), but these assays were not tested on a variety of type strains. Because for the *Leishmania* (*Leishmania*) subgenus ITS2 does not show any advantage over ITS1 and has been less used and evaluated, its potential lies in complementing ITS1 for discriminating species of the *Leishmania* (*Viannia*) subgenus, even though a more extensive validation is required.

(vi) SSU rDNA. The 18S or SSU rDNA has limited use for discriminating *Leishmania* species due to its conserved nature within

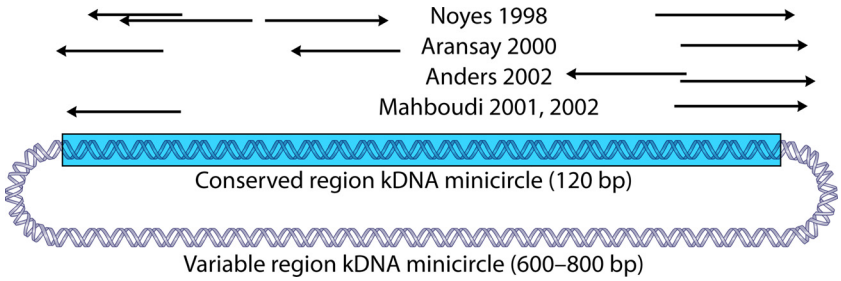

FIG 6 Primers used for species discrimination by size differences of the variable region of kDNA minicircles (134–138). The primers are mapped to a composite sequence obtained from GenBank accession numbers AF308685 and JF831926 (*L. major*). The arrows indicate the 5′-to-3′ orientation. The variable region is not drawn to scale.

the genus. Single-nucleotide polymorphisms have been exploited to identify the *L. mexicana* and *L. donovani* complexes and the *Leishmania* (*Viannia*) subgenus. Methods (Fig. 4) included probe hybridization (121, 122), specific PCRs (123), RFLP analysis (124), and sequencing (24, 77, 124–126). The SSU rDNA region, however, has many merits for first-line diagnostic purposes that need to detect the genus (e.g., see references 124, 127, and 128).

kDNA

Minicircles. Several kinetoplast DNA (kDNA) assays have been described for both the Old World and the New World. In contrast to most other targets used for species typing, the minicircles are not encoded on the parasite's chromosomes but are part of a dense extrachromosomal DNA network called the kinetoplast. This organelle contains the mitochondrial DNA and is found at the base of the flagellum in the order Kinetoplastida to which *Trypanosoma* also belongs (129). The kinetoplast is composed of mini- and maxicircles.

Minicircles are small circular molecules of around 800 bp, containing a conserved region of 120 nucleotides and a highly variable region coding for guide RNAs involved in editing maxicircle genes (130). The general advantage of using minicircles in diagnostics is their high copy number (10,000 copies per parasite), which results in a high sensitivity of assays based on this target. However, minicircles within one strain are not all identical, and they can be divided into minicircle classes (131–133). Because this variability complicates sequencing, most assays for identifying *Leishmania* subgenera and species are based on size discrimination, hybridization with specific probes, or species-specific PCRs.

(i) Size discrimination. Several authors have used size differences of the variable region as a basis for typing of species in the Old World (Fig. 6). The assay of Noyes et al. (134) is widely used, together with those of Aransay et al. (135) and Anders et al. (136). These PCR assays amplify the variable part of the *Leishmania* minicircles (Fig. 6), but many species have minicircles of the same size (Table 1). In all studies, however, *L. major* can be separated from *L. tropica-L. infantum-L. donovani*. Whether *L. aethiopica* can be distinguished based on size alone remains to be established. Aransay et al. (135) complemented size analysis with sequencing of the obtained PCR products, but given the variability of minicircles in a single parasite, and even more so within all strains of the same species, sequence analysis of the variable region is difficult.

Other authors used different primers essentially amplifying the same region (Fig. 6), and included a more extensive panel of New World isolates (137, 138), but, again, only some groups could be separated (Table 1). Even though all PCR primers used in these studies can be mapped to the same minicircle sequence (Fig. 6), it is striking that not all authors could discriminate the same groups on the basis of size (Table 1). This can be explained by the fact that different strains and gel resolutions were tested in each analysis, but it may also be due to amplification of other minicircle classes in different PCRs, as the primers do not have perfect sequence identity with the depicted *L. major* minicircle. Hence, no standardized assays are yet available. In short, kDNA size variation has no global use for separating isolates to the species level and can only be applied in a regional context when certain groups of species need to be discriminated.

(ii) Hybridization. Both partial and complete minicircles and oligonucleotide probes have been used in hybridization assays, either to discriminate the *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) subgenera or to distinguish particular species (e.g., see references 132 and 139 to 143). Most assays rely on hybridization of PCR-amplified minicircle products, even though some have applied the method directly on clinical samples (141), on pelleted *Leishmania* cells (140), or on total *Leishmania* DNA (142). Only the assay of Brenière et al. (139) has been tested on a larger panel of New World parasites and could separate some species complexes (Table 1). The probes of Fernandes et al. (140) could identify the subgenus *Leishmania* (*Viannia*) but were tested on only 8 strains representing 7 species. Nevertheless, they were used by several authors without further validation (e.g., see references 144 and 145). None of the available minicircle hybridization assays has been validated properly to allow separation of all species in either the Old or the New World. Assays often discriminate only to the subgenus level or, at best, identify a few species or species complexes.

(iii) Species-specific PCRs. Sequence polymorphisms in the minicircle kDNA have been exploited to design PCR primers that specifically amplify a species or group of species (e.g., see references 104, 123, 146, and 147). Only the specific primers of Meredith et al. (123) discriminated 4 Old World species, but that assay was validated on one strain of each species only. Several authors have observed cross-reactions between different species (104, 147), which is probably caused by the use of slightly different reaction conditions across labs and the variability of the minicircle population in each species. De Bruijn and Barker (148) reported a widely used *Leishmania* (*Viannia*)-specific PCR, validated on one strain each of 10 species, but cross-reaction of one primer with

human and mouse DNAs was found (149), which could lead to false-positive results. Also, Lopez et al. (150) reported a PCR that amplifies several *Leishmania* (*Viannia*) species but which was evaluated on only 5 strains.

(iv) RFLP analysis. Several reports have described RFLP analysis of the variable minicircle region as an aid in species typing. The obtained fragment patterns form the basis of so-called schizodeme identification (132, 134, 151). In our experience, such patterns are extremely difficult to reproduce, even within the same lab (152), and they have more use in strain tracking or studying intraspecies variability (e.g., see references 62, 153, and 154). Volpini et al. (155) described an RFLP-based species discrimination test based on the conserved region of the minicircles, but only to discriminate *L. braziliensis* from *L. amazonensis*. Rocha et al. (147) further evaluated this RFLP method and demonstrated that *L. lainsoni* and *L. infantum* could also be identified.

(v) Melting curve analysis. Nicolas et al. (156) used differences in melting temperature of PCR-amplified variable and conserved minicircle regions to differentiate some species in the Old World. Their assay was validated on only one or a few type strains from four species and could not separate *L. tropica* from *L. donovani*. The conserved region allowed Pita-Pereira et al. (157) to differentiate a few type strains of *Leishmania* (*Leishmania*) from those of *Leishmania* (*Viannia*), but species identification was not possible. Weirather et al. (104) evaluated several primer sets that could discriminate groups of species.

(vi) LAMP. Even though it is not a PCR procedure, loop-mediated isothermal amplification (LAMP) also constitutes an amplification technology (158). Unlike PCR, it does not require a thermocycler, and the amplified product is visualized in the reaction tube and seen by the naked eye, not requiring gels or fluorescence detection. Even though the technique has been used primarily in first-line diagnostics for parasite detection (159, 160), an *L. donovani*-specific assay based on kDNA minicircles was developed (161, 162). It was tested on only a few type strains for some species, and hence it currently has limited validity.

In conclusion, minicircle kDNA assays show poor performance for separating *Leishmania* species, mainly due to the lack of proper validation and the problem of extensive variability within single strains, and even more so within an entire species. Current methods are not standardized and not usable on a global scale for discriminating to the complex or species level. kDNA minicircle methods seem to be restricted primarily to separating the *Leishmania* (*Viannia*) and *Leishmania* (*Leishmania*) subgenera, and some assays can be used in a regional context for discriminating between species.

Cytochrome *b* (maxicircles). The *Leishmania* cytochrome *b* gene (*cytB*) is encoded on the kDNA maxicircles, of which an estimated 25 to 50 copies are present in each cell, making it a sensitive target for analysis of clinical samples without the need for culturing. Sequencing of the *cytB* coding region has successfully separated most tested species (82, 163), and PCRs were applied directly to clinical and sand fly samples (Fig. 7A) (164–167). Figure 7B shows a dendrogram for a fragment covered by these PCRs, by which most species can be discriminated (Table 1). *L. donovani* and *L. infantum* cannot be separated, nor can *L. braziliensis* and *L. peruviana*. *L. guyanensis* can be separated from *L. shawi*. Sequence analysis of the gene is straightforward, as no size differences were observed, which facilitates sequence alignment. Some *cytB* real-time PCRs were evaluated by Weirather et al. (104), but they could identify only *L. tropica*.

cytB is one of the few genes of *L. siamensis* that have been sequenced, and the species is readily identified from sequence dendrograms (Fig. 7B) (22). In all, *cytB* is a good typing target, rendering a good resolution for all tested species across the globe, is sensitive enough for use on clinical and environmental samples, and is easily analyzed. Sequence validation on a more extended strain panel would further increase its reliability.

Antigen Genes

GP63. The metalloprotease glycoprotein 63 (GP63) is the major surface glycoprotein of *Leishmania* and is considered an important virulence factor and a strong immunogen (168). The gene is arranged as a tandemly repeated unit, and both the intra- and intergenic regions have been used for typing by RFLP analysis. Because of its multicopy nature, assays based on this gene array have been found to be sensitive enough for application directly on clinical samples, without the need for culturing. One report describes a sensitivity of 85% compared with that of kDNA minicircle amplification (169).

For the New World, different fragments were evaluated (169, 170), whereby all tested species could be separated (Table 1). For the Old World, intra- and intergenic *gp63* regions have been used primarily for looking at intraspecies variability (171–174), even though all species could be distinguished (Table 1). Nevertheless, a proper validation with several strains of each species is lacking. Although RFLP analysis seems to be successful for species identification, the obtained patterns can be complicated and difficult to interpret because of so-called isogenes. These are variations between copies within the same parasite (169, 175), which also render sequencing of the *gp63* target impractical. Also, because antigen genes are under constant pressure from the immune system (176, 177), they are primarily suited for exploring clinical pleomorphism at the intraspecies level rather than for typing species.

CPB. Cysteine proteinase B (CPB) is another antigenic protein, and several copies of the gene are present in the *Leishmania* genome, arranged in a tandem array (178). The copies are not all identical and are classified into various subgroups (179–182). Like GP63, it is an important factor in the host-parasite relationship (183) and is therefore especially fit for looking at population structure in a clinical context.

Both the coding sequence and the intergenic regions between the gene copies have been used for typing purposes, primarily using PCR-RFLP approaches to distinguish strains below the species level (e.g., see references 174, 178, and 184). Quispe-Tintaya et al. (178) included in a study of the *L. donovani* species complex a few outgroups that resulted in discriminant RFLP patterns, but *L. tropica* was not tested, nor was the intraspecies variability in *L. aethiopica* and *L. major* (Table 1). PCR-RFLP analysis was also used in the New World (184), but not all species could be amplified, and only *L. braziliensis*, *L. peruviana*, and the *L. guyanensis* complex were identified (Table 1). These PCR-RFLP assays have been used on clinical samples, without culturing (64, 185). In summary, however, none of the *cpb* PCR-RFLP methods have been well validated for species typing. Because the *cpb* array is made up of nonidentical isogenes (178), RFLP patterns are often complicated and vary within the same species.

Some authors have used species-specific *cpb* PCRs that amplify

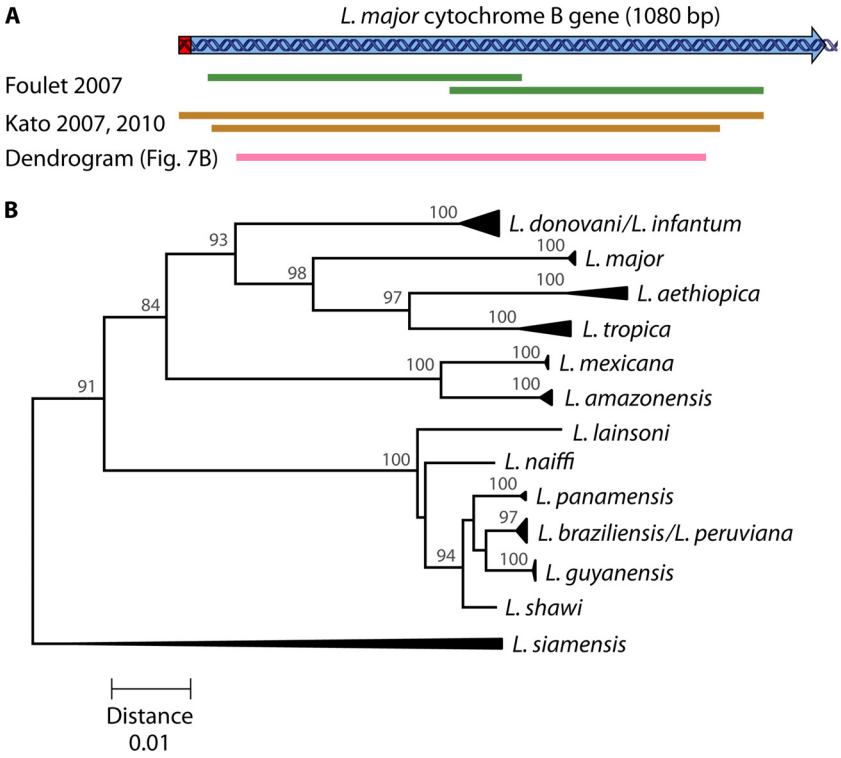

FIG 7 (A) Cytochrome *b* coding sequence of *L. major* (GenBank accession number AB095961) (163). The editing region is shown in red, and the arrow indicates the sense direction. The two overlapping fragments sequenced for typing of clinical samples, covering 887 bp in total, are depicted in green (164). Other fragments amplified for typing of both clinical samples and sand flies are shown in brown, the smallest of which (817 bp) is sequenced (165). The larger brown fragment is the outer PCR amplicon in a nested PCR approach needed for human sample analysis (166). (B) Dendrogram constructed on the basis of cytochrome *b* gene sequences of reference isolates reported by Luyo-Acero et al. (163), Asato et al. (82), Foulet et al. (164), and Leelayova et al. (22), complemented with the sequences under GenBank accession numbers AB433279, AB433280, AB433282, AB566382, AB566381, and AB566380. The analyzed fragment is indicated in panel A (pink fragment). The dendrogram was constructed by the neighbor-joining method and is based on uncorrected *p*-distances (the scale is shown below, in substitutions per nucleotide). Bootstrap values from a 2,000-replicate analysis are depicted in percentages at the internodes, when higher than 70%. The dendrogram was constructed with the software package MEGA5 (253).

one or a few species (179, 181, 186). Lopez et al. (187) developed a more general assay, based on specific PCR amplification of certain groups followed by RFLP analysis to discriminate species within each group, but the assay was developed *in silico* and was not validated on type strains or compared with other methods. Given the complexity of the *cpb* locus, this is a major flaw. Because all these specific assays target only one particular copy of the *cpb* gene array, their sensitivity is reduced, and the PCRs have been applied only for typing of cultured parasites, not directly on environmental or clinical material. A more sensitive LAMP assay was developed by Chaouch et al. (188) for the detection of the *L. donovani* complex, but its specificity was validated on a limited set of type strains.

HSP70. Heat shock protein 70 (HSP70) is also a *Leishmania* antigen, playing a role as a molecular chaperone in protein folding and transport (189). Between 5 and 10 copies of the gene are present in the *Leishmania* genome (190), and minor differences between them may exist in a particular strain (66). Fraga et al. (191) studied *Leishmania* evolution based on *hsp70*. Because many studies have illustrated the value of the heat shock protein 70 gene for species discrimination, it has been used by various researchers (9, 22, 64, 192, 193).

Garcia et al. (Fig. 8) (194) were the first to exploit the variability of the gene for species discrimination in the *Leishmania* (*Viannia*) subgenus on the basis of RFLP analysis. Several authors (67, 195, 196) increased the number of restriction enzymes to allow typing of all *Leishmania* species, irrespective of origin or subgenus (Table 1). Alves da Silva et al. (Fig. 8) (197) evaluated the target on all species circulating in Brazil, using both RFLP analysis and sequencing. This study was further complemented by Van der Auwera et al. (60, 66), who reported over 200 sequences from across the globe and from all medically relevant species (Table 1). All studies showed a nearly perfect congruence with MLEE typing, which makes *hsp70* the most widely validated species typing target to date.

Even though the original PCR of Garcia et al. (194) was used on both clinical and sand fly samples (198, 199), more sensitive PCRs have been developed, some of which amplify shorter fragments (Fig. 8). These were applied in Brazil (200), Peru (201), and several Old World countries (202) and on various clinical sample types. The sensitivities of these assays have not been compared systematically to those of other species typing PCRs but were generally found to be 60 to 90% compared to those of higher-copy-number targets, such as rRNA genes or kDNA minicircles, which are used for diagnostic *Leishmania* genus detection.

Compared to some other targets, *hsp70* combines the possibility of accurate typing to the species level over the entire genus with the ease of sequence or RFLP analysis, as hardly any size variation

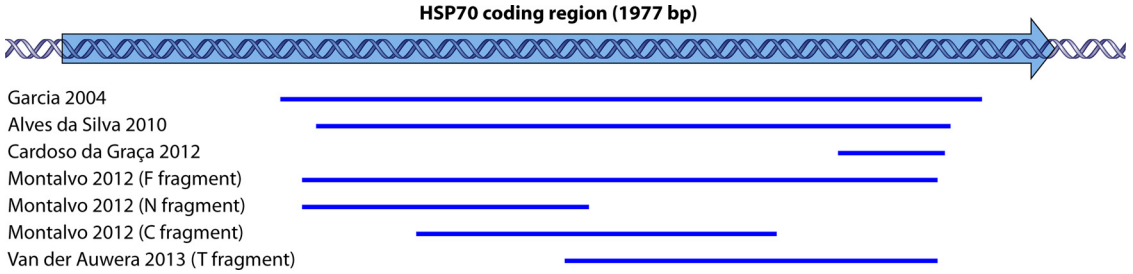

FIG 8 Mapping of *hsp70* fragments used in different studies to the complete coding region of the HSP70 gene (GenBank accession number XM001684512.1) (the arrow indicates the sense direction). These regions were used in either RFLP or sequence analysis (66, 194, 196, 197, 200).

is observed and almost no intraspecies variation is present in currently used RFLP assays. Some authors have developed species-specific PCRs (203). Finally, Requena et al. (204) used the 3′-untranslated region of the gene to distinguish species complexes based on size differences, RFLP analysis, or sequencing (Table 1), but this was not tested on clinical samples.

Miniexon

The miniexon or spliced leader (Fig. 9) is tandemly repeated in the *Leishmania* genome. The exon encodes an RNA that is added to the 5′ end of each protein-encoding RNA during maturation. Even though the exon and intron are conserved in all *Leishmania* species, nontranscribed spacers are variable in both size and sequence (205). Around 100 to 200 copies are present in each genome, which makes the target sufficiently sensitive for analyzing tissue samples (e.g., see references 113 and 206 to 208).

Even though some species groups can be discriminated based on size only (60, 132, 147), RFLP analysis is needed for discriminating species. The PCR (Fig. 9) and RFLP scheme of Marfurt et al. (209) was validated on 12 species, 8 of which could be typed to the species level, with the remaining 4 identified to the complex level (Table 1). Nevertheless, RFLP patterns varied within the same species, and up to 5 restriction endonucleases were required. The assay is therefore quite complicated and can be cumbersome (113). In addition, most species were analyzed based on a single strain, which precludes assessment of the full range of intraspecies variability.

Recently, Van der Auwera et al. (60) carried out a sequence analysis of the same PCR-amplified region on a larger global panel of strains (Table 1). Based on that analysis, the miniexon could discriminate all species from both the Old and the New World, 4 of which were identified only to the complex level. Roelfsema et al. (113) found the sequencing approach to be more practical than RFLP analysis for typing of clinical samples. Nevertheless, sequencing of the miniexon was sometimes problematic due to differences between copies in the same genome and to sequence features, such as homopolymer stretches, causing technical difficulties (60).

7SL-RNA

The 7SL-RNA is an RNA molecule of 250 to 300 nucleotides that plays a role in the translocation process of proteins across the endoplasmic reticulum. Because of its variability and abundance, Zelazny et al. (210) proposed sequencing of a 140-bp PCR-amplified product for typing purposes. They tested the approach on 30 strains, and their analysis was complemented by Van der Auwera et al. (60), using a larger panel of 71 isolates (Table 1). These sequences allowed separation of species complexes rather than individual species, except for *L. major* and *L. lainsoni*, which could be typed individually.

Stevenson et al. (211) extended the region to obtain a 385-bp fragment allowing better discrimination for both subgenera, even though this improved method was validated on fewer strains (Table 1). Nasereddin and Jaffe (103) designed a high-resolution melting assay for the Old World by using a 119-bp fragment of the gene. The assay was tested on a large panel of strains and could differentiate them to the complex level, but *L. aethiopica* was not evaluated (Table 1). Both methods have been used on environmental and clinical samples (212–214).

Carbohydrate Metabolism Enzymes

Glucose-6-phosphate dehydrogenase (G6PDH or G6PD) has been exploited to discriminate between the subgenera *Leishmania* (*Viannia*) and *Leishmania* (*Leishmania*), and within *Leishmania* (*Viannia*) to distinguish the *L. braziliensis* complex, including *L. peruviana* (215, 216). These conventional and real-time specific PCR assays were validated mainly on Brazilian strains, and intraspecies variability was only moderately taken into account (Table 1). They therefore have limited general use. Since they are based on single-copy genes, their sensitivity is lower than that of other methods based on multicopy targets, such as ITS1, *hsp70*, and kDNA minicircles (200), but they have been applied to clinical specimens (30).

Mannose-phosphate isomerase (MPI) has been used in PCR assays to separate *L. peruviana* from *L. braziliensis*, either with a specific PCR (217) or by RFLP analysis (167), even though it is

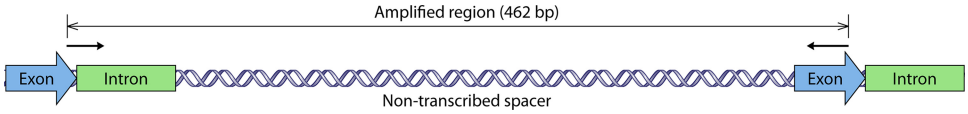

FIG 9 Miniexon repeat of *L. major* (GenBank accession number X69449). The nontranscribed spacer varies in length and sequence between species, from 51 to 341 bp (209). The PCR product amplified in the assay of Marfurt et al. (209) is indicated, whereby primers are shown with arrows in the 5′-to-3′ direction. All regions are drawn to scale.

unclear whether the latter was checked for intraspecies variability. These assays can thus be deployed as a second line of testing when the *L. braziliensis* species complex is identified by G6PDH analysis, and they have been applied to clinical samples (64). Another enzyme downstream in the glycolysis pathway, 6-phosphogluconate dehydrogenase (6PGDH or 6PGD), was also exploited for *Leishmania* typing (5, 218), but only a few Old World species were tested.

Glucose phosphate isomerase (GPI) was applied to discriminate the *Leishmania* (*Viannia*) subgenus and some species complexes in the *Leishmania* (*Leishmania*) subgenus (219). Real-time PCR could be used on clinical samples and was based on specific amplification. It was not evaluated on all species, however, and whether intraspecies variability was properly evaluated is not clear (Table 1). In addition, Weirather et al. (104) used the target in an evaluation of real-time PCR and melting assays.

A combination of carbohydrate metabolism genes was deployed by Tsukayama et al. (68), who combined 6PGD, MPI, and glucose-6-phosphate isomerase with malate dehydrogenase to identify species of the *Leishmania* (*Viannia*) subgenus (Table 1). Single-nucleotide polymorphisms were found for each of the species, but other authors have questioned their general validity (72). They further based two real-time PCRs on melting analysis of fluorescent probes for typing of clinical samples, using *mpi* and *6pgd*. Their assay was evaluated on an extensive reference panel of 64 strains, almost exclusively from Peru. Validation on more strains from other countries would further increase the validity of this technique.

Miscellaneous Targets

Some additional assays have been applied to clinical samples. Piarroux et al. (220) separated all Old World species on the basis of a 222-bp repetitive DNA sequence. This assay was validated on a large sample set (Table 1) and has been applied to clinical samples by using sequence analysis and RFLP analysis (221). Despite these promising results, the method has not been widely applied. Haouas et al. (222) designed a PCR-RFLP scheme based on the topoisomerase II gene for the Old World species, but only 3 reference strains were evaluated, and hence it was not properly validated. Mimori et al. (223) discriminated New World species on the basis of two subgenus- and five species-specific primer sets derived from sequenced randomly amplified polymorphic DNA (RAPD) fragments, but a limited number of strains were evaluated. Weirather et al. (104) designed real-time PCR and melting assays based on a set of genes and could discriminate several species when analyzed on a panel of reference strains. The assays were successfully tested in some clinical samples, but the authors presented no clear and straightforward strategy for general and global use.

Several authors have used a variety of other genomic targets for species typing purposes, but these were not further explored for analyzing clinical samples and were applied to reference strains and cultured isolates only. Strategies include histone 2B RFLP analysis (184); sequencing and RFLP analysis of *N*-acetylglucosamine-1-phosphate transferase (181, 224); PCR and RFLP analysis of hydrophilic acylated surface protein B, also known as K26 (9, 225); glycosomal glyceraldehyde 3-phosphate dehydrogenase sequencing (24); *meta2* RFLP analysis (226); chitinase sequencing (27); sequence analysis of DNA and RNA polymerase genes (227); pteridine reductase 1 sequencing (228); β-tubulin analysis (229); heat shock protein 20 sequencing (230); and sequence analysis of a calmodulin intergenic spacer (231).

Multilocus Typing

Combining different genomic targets has an apparent advantage: one uses information from various loci to gather evidence for a certain species. Such an approach can use either a targeted strategy, whereby several predefined loci are characterized, or a nontargeted approach that randomly documents genome variation. These methods often have limited practical use for species typing, as they provide too much information, which is either redundant or better fit for population-based studies documenting intraspecies variation. When it comes to species typing, multilocus methods are primarily useful for discriminating species belonging to the same species complex, such as *L. infantum-L. donovani* (49–51), *L. guyanensis-L. panamensis* (69, 232), and *L. braziliensis-L. peruviana* (33, 47).

Widely used targeted approaches include MLEE, MLST, and multilocus satellite typing (MLMT). MLEE has already been discussed at length. For MLST, typically several single-copy housekeeping genes are sequenced, and the combined sequence set is used for typing (41, 56, 60, 68, 193). As such, MLST can be considered an upgraded version of MLEE, whereby each enzyme is evaluated by analyzing its coding sequence instead of on the basis of a single gel mobility assessment. Thereby variations that do not affect this mobility are also charted. Even though an MLST outcome is highly robust, the method has no immediate applicability for routine use but rather for establishing the genus's phylogeny (72). So far, it has not been applied to clinical samples: since it is based on mostly single-copy genes, the success rate is expected to be low because of the limited sensitivity. Also, routinely amplifying several genes in parallel and then sequencing them is too costly and time-consuming.

For MLMT (e.g., see references 27, 28, 50, and 233 to 237), several microsatellite regions across the genome are amplified. Microsatellites are repeat regions of di- or trinucleotides, and because the number of repeats is highly variable, the size of the microsatellites makes up a fingerprint pattern. This pattern is used as the basis for typing and studying populations. MLMT has been used on clinical samples (238), but the method requires several PCR amplifications and accurate size analysis. In addition, the method is mainly suited to study variability within one complex, as the markers evolve too rapidly for typing at the species complex level, and markers are complex dependent (28, 72).

RAPD and amplified fragment length polymorphism (AFLP) analyses are both nontargeted strategies. In RAPD analysis, PCR conditions allow permissive primers to hybridize to various nondefined genomic loci, which generates a fingerprint pattern used for species typing (e.g., see references 185 and 239). Due to its nature of permissive priming, the outcome is highly dependent on the exact PCR conditions, making the method unfit for standardization. Variants of the technique have been described for species discrimination, with one specific primer in combination with random priming (240). For AFLP analysis (241, 242), a selection of restriction endonuclease-generated genome fragments is amplified, again resulting in fingerprint patterns that allow discrimination of species (33, 52, 232, 243). However, untargeted methods, such as RAPD and AFLP analyses, are of no use for typing of clinical or environmental samples. As they rely on amplification of random DNA fragments, they are not *Leishmania* specific and

would primarily amplify the abundant host DNA. Hence, they can be applied only to axenically cultured parasites.

Even though the use of multilocus approaches offers the theoretical advantage of sampling information across the genome, in practice single-locus markers render equally valid species typing results, except maybe in the case of simple binary assays (see "Interpreting Species Typing Results." Van der Auwera et al. (60) compared typing outcomes from MLST with those from 4 single-locus methods and found hardly any inconsistencies. Despite the fact that sexual recombination most probably occurs in *Leishmania*, it does not seem to break the clonal population structure observed at the species level (45). As a result, typing from different targets is expected to yield consistent results. This is not the same as saying that typing can be achieved using any locus, as a good typing target needs to fulfill the criteria outlined in "Criteria for Species Typing Tools."

Some authors have used combinations of the single-locus assays described above (e.g., see references 9, 22, 166, 181, 184, and 244). Not all such methods are true multilocus approaches, as they are sometimes deployed in a sequential manner. Veland et al. (64), for instance, discriminated various species based on a predefined algorithm incorporating *mpi*, *cpb*, and *hsp70*, but they used only one locus in each step. Once typing was achieved, the result was not confirmed with a second locus, which in fact makes the method a single-locus approach. In a true multilocus assay, all methods are used in parallel to confirm the detected species.

Detection of Interspecies Hybrids

One possible exception to the general rule that multilocus typing has few advantages over single-locus species determination lies in the identification of hybrid parasites (e.g., see references 30 to 36). Even though recent hybridization can be observed in many loci, alleles from one hybrid parent can be lost over time, and this risk is increased by the highly variable ploidy in natural populations (73). Nevertheless, several examples exist where hybrids were evidenced even in looking at a single locus. For instance, in natural *L. donovani-L. aethiopica* hybrids, both species alleles were present in all genes investigated, including ITS1 and *hsp70* (32, 112), even though this was not recognized at the time of the ITS1 study, which classified the hybrid as an *L. aethiopica* variant. In an *L. infantum-L. major* hybrid, both genomes were present (34), enabling typing of the parasite based on a single-gene assay.

Other examples also exist: for instance, MHOM/PE/2003/LH2538, which was shown to be a hybrid by AFLP analysis, was not recognized as such by *hsp70* analysis (33). As this isolate derived a much smaller proportion of its genome from the undetected parent, such classification is not necessarily problematic. The problem of missing out on hybrids may be an academic one. When a certain hybrid strain has few remnants of the genome of parent 1 in comparison to that of parent 2, typing techniques are more likely to pick up only parent 2 purely by chance. As this parent constitutes the major part of the genome, the parasite likely behaves more like parent 2 than like parent 1, making the typing outcome equally valid. In practice, such a case would be classified as intraspecies variability.

Even though the chance of hybrid detection increases with the number of loci investigated and their locations on different chromosomes, it equally depends on the technology deployed to analyze these loci. SNP analysis, such as RFLP analysis, looks at only a few nucleotides and has a smaller chance of picking up hybrids than does sequencing. In all, given the fact that intraspecies hybrids are rarely documented for *Leishmania*, they do not seem to present a major problem in current typing practices.

Choosing a Tree in the Forest: Which Method To Use?

Selecting the most appropriate species typing technology in a given context can be a challenging task. As illustrated in Currently Applied Methods, below, many different assays have been used, but the question of the basis on which a particular assay is chosen for a given application remains, and this is project rather than lab dependent. Logical factors that can guide the choice of the best technique include technology (what is available in the lab), speed (importance of a short turnaround time), throughput (how many samples need processing in total and simultaneously), personnel (how many hands and brains are available), cost, host (which species are thought to appear in the studied host), location (which species are thought to occur in the region examined), and sample type (cultures, symptomatic infections, asymptomatic carriers, and low or high parasite loads). Especially regarding the presumed species in a particular host or location, one should always keep in mind that this may not be fully understood. In addition, the specifications in "Criteria for Species Typing Tools" should be considered.

One of the items not listed is "history." Researchers often stick with their known territory when species typing is not the main focus of a given project. Often a method is used that was once introduced in the lab but may not be the best choice for the question at hand. Granted, sometimes it can be beneficial to stick with an assay or target with which one has experience, even if it is not optimal in a given situation, but in such a case, at least a critical evaluation should ensure valid results.

In our lab, we have found it a clear advantage to adhere to one particular target for species typing: *hsp70*. Based on this gene, we developed and evaluated both sequencing and RFLP strategies, and this allowed us to build a wide range of experience and a vast range of different methods. We have optimized PCRs for sensitivity and specificity, which makes the target applicable in various settings (66, 67, 191, 194–196, 201, 202). On top of this, several leading labs have developed supplementary *hsp70* assays (9, 197, 200). Confining oneself to one target allows an appreciation of what it can and—at least as importantly—cannot do. When *hsp70* is not able to answer particular questions, other targets are deployed.

In comparing the assays in Table 1, *hsp70* seems to be the best marker for global use, for both New and Old World species and for both subgenera. It discriminates all tested species, including *L. braziliensis* outliers (Fig. 2) and the little-studied *L. siamensis*, and has been tested on more strains from a wide geographical distribution than the case for any other method, for both RFLP analysis and sequencing. In addition, different PCR primers were developed and validated for typing in clinical samples, and sequence analysis is straightforward. Unfortunately, it is not the most sensitive PCR target. Cytochrome *b* has a comparable discrimination efficiency but so far has been evaluated on fewer species and strains, and it relies on sequencing only. Other assays have more restricted use and were designed for either the Old or the New World, or they distinguish fewer species.

Ideally, each lab in the world would base its species typing on the same target, as this would ensure maximal comparability of results from different studies. Because many factors come into

play in selecting the best technique, this will never be possible. Fortunately, outcomes from various methods seem to be largely congruent (60), which is a welcomed consequence of the clonal population structure of *Leishmania*.

Nevertheless, we argue against reinventing the wheel when there is no good reason to do so. New and improved methods should be looked for only when they can give a real added value. In cases where a properly validated and standardized technique is available to implement in a setting or study, there is no need to create yet additional assays.

Interpreting Species Typing Results

In interpreting a species typing result, it is important to keep the limitations of the technique in mind (see also "Criteria for Species Typing Tools"). As further detailed in the next section, many recently published clinical reports specify that species were determined but do not mention the method that was used or mention only that they were determined "by PCR." These papers list no reference or any other information that allows tracing of the applied methodology, illustrating that a species typing result is taken for granted by many. Furthermore, one should always bear in mind that unexpected species may be encountered, such as the *L. major*-like parasites in the New World (Fig. 1), or that patients may be infected by more than one *Leishmania* species (28).

In particular, methods that give a simple binary outcome should be interpreted with caution. Take as an example the species-specific *mpi* PCRs developed by Zhang et al. (217). One PCR specifically amplifies *L. braziliensis* and the other *L. peruviana*, and each test gives a clear outcome depending on whether a product is amplified. However, in many analyses, the dichotomy between both species is not clear-cut, and a large gray zone exists (see "Clinically Relevant Taxa: Certainties and Doubts"). Yet even in this gray zone, each *mpi* assay gives a clear black-or-white result, which can be misleading.

Methods such as sequencing analyze many polymorphisms simultaneously, thereby allowing a more balanced typing outcome. However, in comparative sequence analysis, caution is needed with regard to the selection of a reference sequence set, as several species designations in the public databases have been shown to be erroneous (e.g., see reference 66). Also, a combination of several binary assays gives a smaller chance of errors, as more data are evaluated in such cases. Methods using only a single feature to discriminate species should thus be interpreted with caution, whether the feature is a restriction endonuclease recognition site for RFLP analysis, a specific PCR, a single SNP, a hybridization result, or an antibody binding assay. The chance of misclassification is increased because uncharted mutations may exist, new mutations may arise, or species that were not validated with the method may be taken for other species.

CURRENTLY APPLIED METHODS

General Overview of PubMed Records

Relatively few of the published assays have made it to the field, and the majority have been used primarily in the lab where they were first developed. Such practice makes it difficult to assess their general validity. In order to get an idea of the currently used *Leishmania* species typing methods, we undertook an extensive numerical literature survey based on PubMed records filed between 1 January 2012 and 31 January 2014, with a focus on typing at the subgenus to species levels as explained in "Levels of Typing and Focus of This Review." Papers were filtered based on the title word "leishmaniasis" in conjunction with any of the following search terms: "cutaneous," "mucocutaneous," "mucosal," "tegumentary," "PKDL," or "dermal." The search was performed on 3 February 2014 and returned 410 publications. Of these, 320 were original research papers, both prospective and retrospective. A detailed listing with a summary of each individual analysis can be obtained from the authors upon request. These articles could be categorized into the following 4 groups.

The first group consists of clinical research papers. These 108 papers focus primarily on evaluations of small and large groups of patients and contain reports of clinical, observational, and preclinical studies but not case reports (see below). Of these, 82 were prospective studies. Topics ranged from assessing therapies to evaluation of diagnostic and typing tools, vaccine trials, and descriptive clinical symptomatology, for both humans and canines. In these papers, we looked at the methods used to determine parasite species or subgenus in the patient samples. Many studies also used cultured promastigotes for serologic or protective purposes, but typing of these cultures was not taken into consideration.

The second group, comprising 79 papers, deals with patient management. These are primarily case reports and give an idea of how species typing is performed in actual diagnostic settings. A few papers in this category describe guidelines for management. The third group of publications, 98 in total, are on epidemiology. These papers focus on epidemiological aspects of transmission, reservoirs, vectors, infection, or disease. Not all papers in this category intended to study the parasite or infection of the hosts. Finally, 35 papers dealt with *in vivo* or *in vitro* studies (35 papers), reporting on laboratory analyses of parasites in promastigote cultures, cell lines, or animal models. Such studies used well-characterized strains or clinical isolates, and we assessed whether the reported species were accurately determined to exclude contaminations or culture mix-ups.

Half of the 320 studies did not report any method of typing *Leishmania* at the species level or below the genus level (Table 2). These papers either did not mention any species (34%), relied exclusively on epidemiological data (7%), or used previously documented cultured type strains (9%). The remaining 161 papers reported at least one laboratory method for parasite identification. They also often relied partly on epidemiological information, either because only a subset of samples was analyzed or because the methods discriminated only locally circulating species. In the latter case, it was assumed that no other species had invaded the region under study, implying that the local epidemiology is exactly known.

In the 161 studies that used at least one laboratory typing technique, the different methods and targets were distributed as shown in Fig. 10, whereby several papers reported more than one method. Genetic identification was used in nearly three quarters of all these cases, among which rRNA genes and kDNA minicircles accounted for more than half of the identifications. In 30 reports, the authors used a PCR without specifying a literature reference, technique, or genomic locus, making it difficult to assess whether the typing outcome is valid and illustrating researchers' perception that PCR is a magical technique able to determine a species unequivocally. MLEE, which is still advocated by the World

TABLE 2 Overview of typing methods stratified by study type

Typing method or target	% of studies				
	Clinical[g]	Patient management	Epidemiology	*In vivo* and *in vitro*	Total
No species reported[a]	42 (35)	35	37	0	34
Epidemiology-based studies[b]	14 (16)	1	5	3	7
Type strain-based studies[c]	NA	NA	NA	80	9
rDNA array[d]	7 (7)	11	28	6	14
kDNA minicircles[d]	7 (9)	9	21	0	11
Other genomic targets[d]	14 (16)	11	11	6	12
Unspecified PCR[d]	6 (9)	28	1	0	9
Isoenzymes (MLEE)[d]	7 (6)	4	4	11	6
Antibodies[d]	5 (4)	5	3	9	5
Unspecified[d,e]	6 (9)	6	4	0	5
Total[f]	108 (82)	79	98	35	320

[a] Studies not mentioning any species.
[b] Studies relying only on epidemiology, not using any laboratory-based methods for typing.
[c] Studies relying on previously obtained typing outcomes for cultured strains but not describing any method for confirmation. This category applies only to *in vitro* and *in vivo* studies. NA, not applicable.
[d] Studies using each method for at least one sample. Studies often combined different methods.
[e] Studies using at least one method that was not properly described or referenced.
[f] Total number of publications for each study category. The category in which each individual study was classified can be obtained upon request (see "General Overview of PubMed Records" in the text).
[g] Numbers are for all clinical reports, and numbers in parentheses relate to prospective studies.

Health Organization as the reference method (65), was used in 12% of studies where typing was done. Antibodies were used in 9% of cases, and 10% of studies did not specify any details other than that the species was determined.

Methods in Different Study Types

Table 2 gives an overview of the methods for species typing that were used in the different study types. It is noteworthy that in 42% of clinical studies, the causative species was not reported, and in another 14% of these studies, the authors relied exclusively on epidemiological data. Hence, in fewer than half of the clinical studies (44%) was the identity of the species checked to some extent. This was slightly better in prospective studies, even though typing was done in only 49% of these reports. Based on extensive reviews in the Old World and the New World, Gonzalez et al. (19) found similar numbers, with about 50% of clinical studies actually determining the causative species.

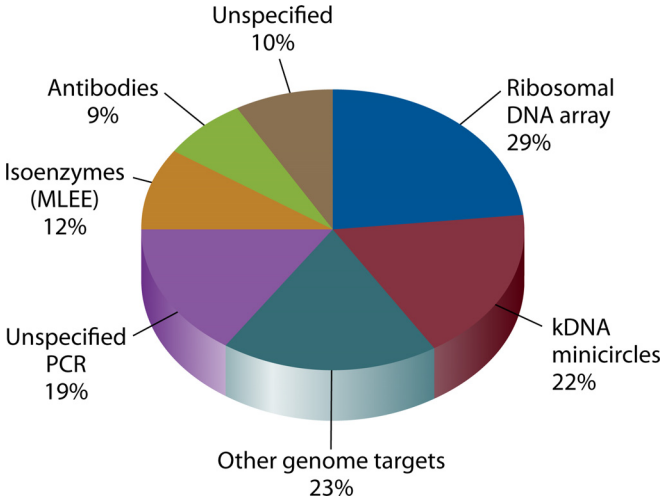

FIG 10 Relative use of different species typing methods and targets in the 161 studies where parasite typing was done. The numbers give the percentages of studies using the different methods. The total percentage is more than 100, as many studies used more than one technique.

Unfortunately, reports that do not mention or check the *Leishmania* species are of limited use outside the area where data were gathered, and in that sense they contribute little to our global understanding of the behavior of species in patients. In recent recommendations on designing good clinical studies, species typing was generally advised because of the possible differential drug efficacy (76, 245). This was also concluded in several meta-analyses of clinical trials of CL and MCL (19, 246). For patient management, 64% of studies indicated the use of species identification methods, even though in such cases it is also considered relevant (16, 247).

In vitro and *in vivo* studies always reported the species of the isolates used in the assays, but rarely did the authors specify whether they included some verification of whether the parasites they used actually corresponded to the reported species. Such practice may result in describing a species different from the one actually studied as a result of undetected culture contamination. Van der Auwera et al. (66) listed several examples of erroneous *Leishmania* species designations in GenBank, with some sequences belonging to a different species complex from the one on file, and even with some being more related to *Leptomonas* than to *Leishmania*. These erroneous classifications are likely the result of culture switches or contamination, which can be avoided by applying adequate typing. As for epidemiological studies, 58% reported *Leishmania* species identification, while the remaining studies focused on different aspects of epidemiology.

With regard to the chosen method, clinical studies relied on a variety of genomic targets, while this was less the case for patient management and epidemiology. The latter made more use of kDNA minicircles and rDNA, which may be related to the routine nature of these labs, where parasite identification has a practical

TABLE 3 Overview of typing methods stratified by region

Typing method or target	% of studies						
	Mediterranean basin	Middle East (Iran)[g,h]	Far East (India)[i]	Africa[g]	Latin America (except Brazil)[j]	Brazil	Countries where leishmaniasis is not endemic
No species reported[a]	34	30 (26)	44 (43)	36	26	46	13
Epidemiology-based studies[b]	4	3 (3)	8 (7)	18	3	15	0
Type strain-based studies[c]	6	5 (5)	5 (7)	0	3	13	23
rDNA array[d]	21	27 (28)	18 (17)	36	6	4	5
kDNA minicircles[d]	2	27 (28)	10 (7)	0	6	11	8
Other genome targets[d]	13	3 (3)	3 (3)	18	31	8	21
Unspecified PCR[d]	11	5 (5)	13 (17)	0	9	3	28
Isoenzymes (MLEE)[d]	6	2 (2)	0 (0)	0	14	9	8
Antibodies[d]	2	2 (2)	10 (10)	0	11	6	0
Unspecified[d,e]	11	5 (5)	3 (3)	0	6	1	8
Total[f]	53	63 (58)	39 (30)	11	35	80	39

[a] Studies not mentioning any species.
[b] Studies relying only on epidemiology, not using any laboratory-based methods for typing.
[c] Studies relying on previously obtained typing outcomes for cultured strains but not describing any method for confirmation. This category applies only to *in vitro* and *in vivo* studies.
[d] Studies using each method for at least one sample. Studies often combined different methods.
[e] Studies using at least one method that was not properly described or referenced.
[f] Total number of publications for each study category. The category in which each individual study was classified can be obtained upon request (see "General Overview of PubMed Records" in the text).
[g] Countries bordering the Mediterranean Sea are excluded here and are listed in the category "Mediterranean basin."
[h] The first numbers include studies from Iran, and numbers in parentheses are for Iran only.
[i] The Far East category includes the Indian subcontinent. The total values are given, and numbers for India alone are given in parentheses.
[j] Mexico and Central and South America, excluding Brazil.

use and less effort is spent on precise (intra)species characterization. Also, labs where clinical trials are carried out often have a more biology-oriented character, and, frequently, different aspects of the parasite are investigated. This may include the study of various genomic loci not commonly deployed in routine settings.

Methods in Different Geographical Areas

Table 3 stratifies the methods used by the locality where they were carried out. The sites where the studies were performed rather than where the samples originated from were considered, which were generally the same for areas of endemicity. From these data, it is apparent that Old World countries relied mostly on rDNA-based methods for species typing, except for the Middle East, where kDNA minicircles were equally popular. Given the fact that studies in these regions focused on the *Leishmania* (*Leishmania*) subgenus, where variability in the rDNA sequences is sufficient to determine the species or species complexes, this is logical. Minicircle kDNA was used particularly in Iran, where 16 of the 22 Old World minicircle-based studies were performed.

In Latin America, the picture is more diverse, and a variety of targets were used. Only in Brazil did kDNA minicircle assays slightly outnumber other techniques, probably for historical reasons, as many minicircle-based assays were developed and validated there. In other countries, only *cpb* and *hsp70* assays were used in more than one lab, illustrating the scattered typing landscape. This relates to the difficulties in efficiently discriminating *Leishmania* (*Viannia*) species, leading to many assay development efforts. In addition, rDNA offers limited possibilities for this subgenus, so it was not frequently deployed.

In countries where leishmaniasis is not endemic but was investigated, no studies relied only on epidemiology, and only 13% did not mention parasite species. A variety of methods were used. In clinical practice, these countries deal with import leishmaniasis (15, 16, 248), and as the site of infection in such cases is not always known, globally applicable typing strategies are needed. Neither minicircles nor rDNA provides such a possibility, so other methods must be sought. In addition, labs in these countries frequently participate in clinical or *in vitro* and *in vivo* studies. For these studies, a detailed parasite identification is frequently needed to correlate with clinical and biological data, relying on several targets or even the entire parasite's genome (e.g., see references 73, 249, and 250).

It is striking that Brazil is among the countries where the fewest efforts for parasite typing were reported, as parasite identification was done in merely 21 of 80 studies. Also, India and the Far East countries scored low, but they have far less parasite diversity. In India, visceral leishmaniasis caused by *L. donovani* is the prominent disease, and diagnosis of PKDL can be established on clinical grounds and patient history. Brazil, on the other hand, is home to 8 *Leishmania* species and an entire spectrum of diseases (2), yet confirmation of the infecting agent is apparently not considered important by several authors, or they rely blindly on epidemiological data.

Limitations of PubMed Analysis

This overview of currently applied *Leishmania* typing strategies is by no means complete. First, it is based only on PubMed records gathered over 2 years, whereby routinely applied techniques in various types of health centers remain hidden because they often are not published in indexed journals. Two countries were responsible for 43% of the original research papers published in the

analyzed period: Brazil and Iran. This is not a coincidence, as both are in the top 4 countries with the highest estimated incidences of cutaneous leishmaniasis (2). Only Afghanistan and Algeria have higher estimated burdens, but each of them published merely one paper in the studied period.

Nevertheless, this literature review illustrates the scattered use of various typing methods and genetic targets. It also gives an idea of how often scientists and physicians rely on epidemiological information, even though species distribution may change over time and is mostly not systematically monitored. Our overview underscores the need to use more standardized and uniform species typing tools, as this would allow a better evaluation of the assays on a global scale and facilitate comparison between different studies. It also highlights the need for accessible but accurate assays in various settings, allowing continuous monitoring of an ever-changing epidemiology.

PARASITE TYPING BEYOND THE SPECIES LEVEL

Parasite identification should not be limited to species determination. Even though particular species can be associated with certain forms of the disease or can affect prognosis and cure, two cases can be envisioned where additional typing is clinically relevant and the development of additional typing techniques is recommended.

First, within each species, different populations are expected to exist. Such populations can have specific characteristics, such as virulence or tolerance to certain drugs, which make their identification relevant. This concept relates to identification of different discrete typing units (DTUs) within a given species or species complex (28). Studying population dynamics in a given geographical location requires methods that are able to expose a higher resolution than those of the methods focused on here. MLMT, MLST, AFLP, MLEE, and RAPD analyses have been used successfully to uncover intraspecies differences. Of these, MLMT has the most potential for use on clinical samples without the need for culturing, because it is sensitive and targeted. Nevertheless, in cases of recent outbreaks, in which strains are genetically very similar, this method is not applicable (251). The most informative method to date is sequencing of the entire genome (e.g., see reference 73), even though this too still requires parasite isolation. A major hurdle when it comes to studying populations lies in the difficulty of examining parasites in wild animal reservoirs, because of their poor accessibility, and in asymptomatically infected individuals, because few parasites can be found. Also, parasite loads can be low in symptomatic disease. These problems all lead to a potential bias in uncovering the complete population structure (28).

Second, certain biological traits may be conferred by genetic mechanisms that surpass the population boundary (1, 28). For instance, strains belonging to different populations or even species may share pathways that render them drug resistant or that enable them to colonize certain cell types or sand fly species. In such cases, if the biological pathway is known or markers correlated with these traits have been identified, characterizing parasites by such features may be more relevant than looking at the species.

RECOMMENDATIONS

Verification of the *Leishmania* species is considered a must in any report describing clinical data and is a valuable aid in day-to-day patient management (16, 19, 76, 245–247). To this end, single-gene sequencing is the most practical method in a clinical (human or animal) context. Several genes render good resolution at the species level, but ITS1 (Old World) and *hsp70* (global) seem to be the best choices in terms of sensitivity and validation, possibly along with the cytochrome *b* gene. The miniexon offers good resolution globally, but the sequencing process is cumbersome. If sequencing is not possible, RFLP analyses of *hsp70*, ITS1 (Old World), and the miniexon may be considered, as these have been used and validated successfully. However, the miniexon RFLP scheme is complicated and suffers from a high degree of intraspecies variability. For species typing in epidemiological and environmental studies, only MLEE has been applied to all currently described species. No other single method has been used on the entire genus. Consequently, additional validation is needed to include species exclusively found in sand fly vectors or animals.

Wherever possible, we advise investigators to adhere to one target, preferably one that others use as well, as this allows one to gain a good sense of what it can or cannot do in a given context. "Reinventing the wheel" is discouraged unless a marked improvement of the existing range of assays can be realized.

In the absence of a clear species border within certain complexes, we advise adoption of the "better sure than sorry" strategy. When sequencing places a parasite somewhere halfway between different species in the complex, it is better to report at the complex than the species level. This rule is difficult to adhere to when using RFLP- or other SNP-based methods, which inherently give a black-or-white answer (see "Interpreting Species Typing Results").

These are our opinions, taking into consideration the current knowledge and flaws. Often practical limitations stand in the way of using the best available technique, and one is forced to find a pragmatic compromise. Ideally, everyone would use the same target for all experiments, but this is not realistic. Full-genome sequencing is the ultimate target for establishing a consensus reference taxonomy, to be mirrored by simpler field-applicable assays. While awaiting such comprehensive analysis, we will have to cope with assays that best match the existing species reference framework for many years to come. This may not be ideal, but it seems to be quite manageable and fairly accurate when it comes to species typing for dermal leishmaniasis.

APPENDIX

GLOSSARY

AFLP Amplified fragment length polymorphism. Fingerprinting technique whereby genomic DNA is digested with a frequent and a rare-cutting restriction endonuclease, after which a selection of the obtained fragments are amplified by PCR and separated based on size (241).

amplicon Amplified PCR product.

CL Cutaneous leishmaniasis. In this review used to refer to MCL, ML, LCL, and DCL.

DCL Diffuse cutaneous leishmaniasis. Characterized by multiple lesions resulting from dissemination of the infection from the inoculation site.

DTU Discrete typing unit.

HRM High-resolution melting. Real-time PCR technique whereby the melting temperature of a double-stranded DNA fragment or bound oligonucleotide probe is determined.

isoenzymes Variations of the same enzyme across different strains. These variations migrate differently in a pH-gradient gel, and the collective pattern of all isoenzymes is called a zymodeme.

ITS Internal transcribed spacer of the rDNA array (Fig. 4).

kDNA Kinetoplast DNA. Extrachromosomal mitochondrial DNA in kinetoplastid organisms, such as *Leishmania*. kDNA consists of a dense network of mini- and maxicircles.

LCL Localized cutaneous leishmaniasis. Characterized by a single nodular or ulcerative lesion at the site of parasite inoculation by the sand fly vector.

MCL Mucocutaneous leishmaniasis. Typical of the New World and generally produced by mucosal metastasis from a primary local cutaneous lesion, often after healing of the latter, even years later.

microsatellite Short nucleotide repeat that varies in size between different strains.

ML Mucosal leishmaniasis. A variant of MCL in which only mucosae are affected, possibly by extension of contiguous facial skin lesions or direct mucosal inoculation by an insect (252).

MLEE Multilocus enzyme electrophoresis. Technique to determine the zymodeme of strains, long used as the gold standard for *Leishmania* species typing.

MLMT Multilocus microsatellite typing. In this technique, several microsatellites in the genome are PCR amplified, and the combination of their lengths allows definition of parasites and parasite populations.

MLST Multilocus sequence typing. Technique whereby several genes are sequenced and the species is determined on the basis of this sequence information. Usually, single-copy housekeeping genes are selected.

PKDL Post-kala-azar dermal leishmaniasis, a dermal manifestation characterized by multiple lesions and following successful treatment of visceral leishmaniasis caused by *L. donovani* (3).

RAPD Randomly amplified polymorphic DNA. Also called permissively primed PCR. Technique whereby primers are allowed to hybridize to nonspecified fragments in a DNA sample, leading to a collection of fragments that together define a fingerprint pattern.

rDNA rRNA gene array (Fig. 4).

restriction endonuclease Enzyme cutting DNA in a sequence-dependent manner. Upon recognizing a specific DNA sequence, the enzyme cuts both strands of the DNA. Each restriction enzyme recognizes a particular sequence, most frequently of 4 or 6 nucleotides.

RFLP Restriction fragment length polymorphism. Method whereby a DNA fragment (in the case of typing; usually a PCR product) is cut by one or a combination of restriction endonucleases. The obtained fragments are separated based on length, resulting in an RFLP pattern that is sequence dependent (Fig. 3).

SNP Single-nucleotide polymorphism. DNA base change in the genome of a particular species or parasite variant.

species complex Group of related *Leishmania* species, also called a "complex." The level of species complex is situated between subgenera and individual species (Fig. 1).

VL Visceral leishmaniasis (kala-azar).

zymodeme Collection of *Leishmania* strains sharing the same isoenzyme profile.

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