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# MINIREVIEWS

# Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications[▽]

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*Leishmania* parasites are the etiological agents of the leishmaniases. The parasites are transmitted to mammals, including humans, by the bite of phlebotomine sand flies and occasionally, by sharing of needles, blood transfusion, and congenital transmission (18). In terms of global burden of disease, the leishmaniases are the third most important vector-borne disease, and it is estimated that worldwide there are an annual 1.5 to 2 million cases, with up to 350 million people at risk of infection and disease.

Surveillance data indicate that the global number of cases has increased in recent decades, and several important epidemics have been reported (e.g., Sudan and Afghanistan). Such increases can be explained, in part, by improved diagnosis and case notification but are also due to other factors such as inadequate vector or reservoir control; increased detection of disease associated with opportunistic infections (e.g., human immunodeficiency virus [HIV]/AIDS), urbanization, and deforestation; the emergence of antileishmanial drug resistance; economic hardship; armed conflict; and tourism. Particularly, the latter two factors have led to the increasing observation and management of leishmaniasis patients in clinical practices in areas where this disease is traditionally not endemic in North America and Northern Europe. Thus, more than 600 U.S. soldiers contracted leishmaniasis in Iraq since 2003, most of which were diagnosed and treated at the Walter Read Army Medical Center in Washington, D.C. (28). Similarly, in the United Kingdom the number of travelers with leishmaniasis seen by the Hospital of Tropical Diseases in London has more than quadrupled in the past 10 years (13). Here we critically review current molecular approaches for leishmaniasis diagnosis, primarily focusing on the detection of human disease rather than their applications in the veterinary field.

## CLINICAL PATHOLOGY

The reason why the leishmaniases are such a diagnostic challenge is because of the wide spectrum of clinical manifestations that they may present: ulcerative skin lesions developing at the site of the sand fly bite (localized cutaneous leishmaniasis); multiple nonulcerative nodules (diffuse cutaneous

leishmaniasis); destructive mucosal inflammation (mucosal leishmaniasis [ML]); and disseminated, potentially fatal, visceral infection (visceral leishmaniasis [VL]) (18). These main manifestations may themselves deviate, complicating definitive clinical diagnosis even further. Cutaneous leishmaniasis (CL) lesions, for example, may vary in severity (e.g., in lesion size), clinical appearance (e.g., open ulcer versus flat plaques versus wart-like lesions), and duration (e.g., in time of evolution or in time to spontaneous cure).

Several *Leishmania* species may cause the leishmaniases in children and adults. The first sign of an infection typically is a small erythema at the site where an infected sand fly has bitten and regurgitated parasites into the skin. Once infection is established, depending on the parasite strain or species, host immunity, and other, as-yet-unidentified factors, parasites will cause an inflammatory reaction that leads the erythema to develop into an open ulcer (localized cutaneous leishmaniasis) or to visceralize to lymph nodes, spleen, and liver (VL).

The range of clinical manifestations observed in leishmaniasis patients is mirrored by the complexity of leishmaniasis epizootiology. Infections are caused by more than a dozen *Leishmania* species, and numerous sand fly and mammal species have been incriminated as vectors and reservoir hosts, respectively.

## PARASITOLOGICAL AND SEROLOGICAL DIAGNOSIS

The broad clinical spectrum of the leishmaniases makes the diagnosis of present and past cases difficult. However, differential diagnosis is important because diseases of other etiologies with a clinical spectrum similar to that of the leishmaniases (e.g., leprosy, skin cancers, and tuberculosis for CL and malaria and schistosomiasis for VL) are often present in areas of endemicity. Also, clinical disease severity is mainly due to the infecting *Leishmania* species, and there is growing evidence that the therapeutic response is species and, perhaps, even strain specific.

**Microscopy and culture.** Parasitological diagnosis remains the gold standard in leishmaniasis diagnosis because of its high specificity (10). This is typically undertaken by microscopic examination of Giemsa-stained lesion biopsy smears (CL) or lymph node, bone marrow, and spleen aspirates (VL). Occasionally, histopathological examination of fixed lesion biopsies or culture of biopsy triturates and aspirates is also performed.

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Microscopy is probably still the standard diagnostic approach at tertiary, secondary, or even primary health levels in areas of endemicity, because more sophisticated techniques are currently expensive and rarely available. Culture in combination with multilocus enzyme electrophoresis allows for parasite species identification and characterization. However, it requires a wealth of technical expertise and is time-consuming, and the results are potentially biased because of the isolation and in vitro maintenance procedures. Importantly, the sensitivity of microscopy and culture tends to be low and can be highly variable (10), depending on the number and dispersion of parasites in biopsy samples, the sampling procedure, and most of all the technical skills of the personnel.

**Immunological techniques.** Several serological approaches are commonly used in VL diagnosis. In particular, freeze-dried antigen-based direct agglutination tests and commercially available immunochromatographic dipstick tests have increasingly become reference tests in operational settings since they have high sensitivity and specificity (3), are easy to use, and require minimal technological expertise or laboratory setup. Serological tests are rarely used in CL diagnosis because sensitivity can be variable and because the number of circulating antibodies against CL-causing parasites tends to be low (e.g., if previous chemotherapy has been administered). The specificity can also be variable, especially in areas where cross-reacting parasites (e.g., *Trypanosoma cruzi*) are prevalent.

The Montenegro skin test (MST) is occasionally used in CL diagnosis (e.g., in epidemiological surveys and vaccine studies) because of its simple use and because of its high sensitivity and specificity (26). The main disadvantages of the MST are that it requires culture facilities to produce the MST antigen, that different antigen preparations impact test sensitivity, and that the test does not distinguish between past and present infections. The MST is not used for VL diagnosis, since patients only develop strong *Leishmania*-specific cell-mediated immunity when cured (1).

## MOLECULAR DIAGNOSIS

**Available molecular approaches.** Although different molecular methods have successively been evaluated for leishmaniasis diagnosis (e.g., pulsed-field gel electrophoresis and multilocus enzyme electrophoresis), PCR-based assays currently constitute the main molecular diagnostic approach of researchers and health professionals. Several distinct PCR formats are available that may broadly be classified into "mid-tech," "high-tech," and "low-tech" approaches. Mid-tech approaches are probably the most widely used, and comprise conventional PCR assays, in which PCR amplicons are resolved by electrophoresis (eventually after cleavage with restriction enzymes, i.e., PCR and restriction fragment length polymorphism analysis [PCR-RFLP]) and visualized after ethidium bromide staining (23). These assays are performed with several pieces of laboratory equipment (e.g., a thermocycler, a power supply, an electrophoresis tank, a UV transilluminator, and a camera) available in any standard molecular laboratory and are generally time-consuming (which may considerably alter the cost of analysis, depending on the personnel costs). High-tech approaches are methods in which PCR products are analyzed during their amplification (so-called real-time PCR) after

staining with SYBR-green I dye or hybridization with fluorogenic probes (e.g., TaqMan or fluorescence resonance energy transfer [FRET]) (7). In this case, assays are performed with a single all-in setup, and the detection of fluorescence is done within a closed tube, decreasing the risk of laboratory contamination by amplicons. Applications are rapid and of high-throughput, but equipment is comparatively expensive, and working costs remain high (e.g., according to our own estimation at the Instituut voor Tropische Geneeskunde, Antwerp, Belgium, the cost per sample analyzed is $12 [U.S. dollars] for FRET-based assays versus $2.5 for PCR-RFLP). Low-tech approaches refer to simplified PCR methods for use in laboratory settings with minimal equipment. Simplification can potentially be done at the two main steps of the PCR protocol: target amplification and detection of the PCR products. Loop-mediated isothermal amplification (LAMP) represents a promising avenue for both steps: it requires only a simple water bath for amplification, and detection can be done visually by using SYBR-green I dye, which turns green in the presence of amplified products and remains orange in its absence. The method was claimed to be 100 times more sensitive than conventional PCR in the detection of *Trypanosoma brucei* (11), but thus far, this has—to our knowledge—not been applied to leishmaniasis diagnosis yet. Simplification of detection has been attempted by PCR-enzyme-linked immunosorbent assay (ELISA), a "reverse hybridization" method based on the capture of PCR amplicons by specific probes immobilized in ELISA microtiter wells and colorimetric visualization (6). High sensitivity was observed in blood samples from HIV-negative VL patients (6). However, in PCR-ELISA, detection is still dependent on sophisticated equipment (i.e., an ELISA plate reader). More recent methods, such as oligochromatography-PCR (OC-PCR) (5), represent a more promising alternative. This method requires a PCR cycler and a water bath, and PCR products are visualized in 5 min on a dipstick through hybridization with a gold-conjugated probe; an additional advantage is that internal PCR controls can be placed onto the dipstick. Phase I evaluation of a first OC-PCR prototype for the diagnosis of sleeping sickness revealed 100% sensitivity and specificity (5); a similar prototype is currently under evaluation for the diagnosis of leishmaniasis (see http://www.tryleidiag.org). Combination of concepts such as LAMP and OC should be explored and would represent a significant development of low-tech molecular assays and a step to the democratization of molecular diagnostics to resource-constrained countries, if reagent costs can be kept to a minimum.

**Practical applications of molecular methods in leishmaniasis diagnostics.** In terms of practical applications, six main clinical and/or biological questions may be answered by nucleic-acid based methods to diagnose leishmaniasis.

First, PCR allows a highly sensitive and specific (up to 100%) detection of the *Leishmania* parasite irrespective of species or genus. This application is required for differential diagnosis before initiating therapy, and the performances of PCR have consistently been shown to be better than microscopy or parasite culture, particularly in samples with low parasite loads (e.g., in ML patients [9]) or in samples from less intrusive sources, such as blood (4) and conjunctiva (24). The contribution of PCR also appears to be particularly relevant for the diagnosis of leishmaniasis in patients coinfected with

HIV (2, 4, 6). Parasite detection by PCR for confirmation of a clinical cure appears to be important in VL (17) but should be further explored in CL since up to 80% of patient scars remain PCR positive, even 8 years after their clinical cure (22).

Second, host tissue quantification of parasites might be assessed by PCR. This may be highly relevant for monitoring disease progression and outcome of antileishmanial therapy, e.g., for the clinical management of HIV-coinfected patients (2) and those cured CL patients at risk of developing ML. For such application, protocols of real-time quantitative PCR amplification of DNA have been developed, which reportedly have high analytical sensitivity (0.0125 parasites per ml of blood) and excellent linearity (14).

Third, for some applications, it might be necessary to demonstrate the viability of the detected parasites, e.g., when assessing the efficacy of drug therapies and predicting treatment outcomes. In this case, RNA should be preferred to DNA as an amplification target because the latter is still detected for a long time (estimated at 24 h) after parasite death (12). RNA quantification could be done by reverse transcription real-time PCR, but alternative protocols are available (e.g., quantitative nucleic acid sequence-based amplification [QT-NASBA]) (25). QT-NASBA allows isothermal nucleic acid amplification of target sequences by the simultaneous enzymatic activity of avian myeloblastosis virus reverse transcriptase, T7 RNA polymerase, and RNase H. Targeting RNA might also bring operational advantages: compared to DNA, the starting number of template molecules is much higher (particularly for rRNA), which might significantly increase assay sensitivity and decrease required sample volume.

Fourth, *Leishmania* species identification can be performed by a series of PCR-based assays. This is useful for the clinical management of the leishmaniasis patients, because of the established link between some *Leishmania* species and (i) disease severity and (ii) treatment outcome (18) (see also http://www .leishnatdrug.org). Several high-tech and mid-tech molecular assays are available, such as the FRET-melting curve analysis for molecular typing of the *Leishmania donovani* complex (19), the PCR-RFLP analysis of *Leishmania* hsp70 genes (9), and/or single nucleotide polymorphism analysis of metabolic enzyme genes (16). Low-tech assays are currently in the pipeline, including a reverse line blot-PCR (C. Jaffe, unpublished data) or oligochromatography (T. Laurent and G. Van der Auwera, unpublished data).

Fifth, molecular diagnosis might allow defining parasite-specific features such as virulence or drug resistance. This application is still being explored since it requires identifying robust markers of the corresponding phenotypes (not yet available), but it might be relevant in the future for the prognosis or determination of the most adequate treatment or identification of those patients at risk of ML.

Sixth, highly discriminatory fingerprinting tools might be useful for so-called "parasite tracking." Such an application is probably more relevant for epidemiological purposes than for diagnosis (e.g., in outbreak investigations or tracking drug-resistant parasite strains). The best illustration of the performances of fingerprinting comes from the work of Cruz et al. (4), who analyzed the kinetoplast DNA RFLP patterns of *Leishmania* parasites in syringes discarded by intravenous drug users, demonstrating that syringe sharing can indeed promote the spread of *Leishmania* clones among intravenous drug users.

In real life, these six molecular applications will be implemented depending on several criteria, including the clinical relevance of the corresponding hypothesis to be answered, the availability of alternative methods, the technical skills of the personnel, and/or the extent of the laboratory setup. For example, for simple parasite detection in clinical laboratories of countries where the parasite is not endemic the trend is to prefer molecular diagnosis, because microscopists with extensive experience in detecting amastigotes in microscopy slides are usually not available and laboratory facilities are well equipped. In contrast, in countries where the parasite is endemic, where microscopists' skills are maintained due to routine laboratory practice, microscopy tends to be preferred as the first-line parasite detection method. Obviously, for the five other applications outlined above, there is no alternative other than nucleic acid-based methods, which further highlights the need for the development of low-tech PCR methods.

**Operational aspects.** The choice of a specific PCR assay and approach will depend on two main criteria. On one hand, high-throughput applications (e.g., central reference laboratories or epidemic outbreak investigations) would require methods such as real-time PCR or PCR-ELISA, whereas single-test applications (e.g., peripheral or travel medicine laboratories) could be done with other assays (e.g., oligochromatography). On the other hand, according to the local infrastructure, training, and budget, low-tech assays could be preferred to high-tech ones, when available (Table 1).

Clearly, the applicability of PCR in the six approaches outlined above depends on the existence of adequate genetic markers. For detection, quantification, and viability studies, in which sensitivity must be maximized, high-copy-number targets are chosen (e.g., rRNA genes, kinetoplast DNA minicircles, or mini-exon genes [1]). For species identification, both sensitivity and discrimination at a given taxonomic level are required and, hence, repeated and polymorphic sequences are targeted (e.g., gp63, rRNA gene-internal transcribed spacers, hsp70, and cysteine proteinases) (9). For parasite tracking, where fingerprinting is needed, the resolving power must be high, and targets such as kinetoplast DNA, microsatellites, or some antigen-encoding genes are used.

A major concern in the research and development and in the implementation of molecular assays is the lack of standardization and quality control. A crude search in the PubMed database revealed that, to date, more than 400 publications on PCR diagnosis of leishmaniasis have been published since 1989, in which a multitude of gene targets, protocols, and applications are described. Surprisingly, only a few studies have compared different available protocols, either for biopsy sampling (e.g., dermal scrapings taken from the bottom of the CL lesions versus scrapings from the margin of CL lesions [20], biopsy samples versus scrape-exudate versus syringe-sucked fluid of CL lesions [15]), DNA extraction (e.g., phenol-chloroform versus commercial kits [21]), or the use of PCR primers (e.g., kinetoplast versus rRNA gene primers [1, 20, 21, 26]) (Table 1). The findings of these comparative studies might differ from one report to another: for example, whereas one study indicated a higher sensitivity of kinetoplast DNA-based assays (1), another reported a sensitivity similar to those based on rRNA

TABLE 1. Practical guide for the molecular diagnosis of leishmaniasis

| Step | Description |
| --- | --- |
| Overall | A myriad of PCR protocols to detect *Leishmania* DNA in clinical samples have been developed. Depending on the laboratory setup, the origin of clinical samples, sample storage, and processing, the DNA extraction protocol, the choice of PCR primers, and the PCR methodology protocols can vary considerably in sensitivity and specificity. One of the main drawbacks of research to date has been the absence of multicenter studies evaluating PCR protocols for leishmaniasis diagnosis. Most of the diagnostic PCR protocols for leishmaniasis are not validated "in-house" protocols, also known as "home-brew" assays. Only one, a protocol developed by the Walter Reed Army Institute of Research (WRAIR) based on the original work of Wortmann et al. (27), is approved by the College of American Pathology (CAP), with the WRAIR laboratory becoming CAP certified for leishmaniasis diagnosis using this specific protocol. A new, "second-generation" assay using a drydown bead format and performed using SmartCycler technology (http://www.cepheid.com) is currently being validated in support of a Food and Drug Administration application expected in early 2007 (A. Magill, unpublished data). |
| Sample collection | PCR protocols to detect *Leishmania* DNA have used a variety of samples, including lesion biopsies, scrapings, imprints, smears, exudates, and aspirates (CL and ML diagnosis); spleen, lymph node, and bone marrow aspirates (mainly in VL diagnosis); conjunctival swabs and aspirates (VL diagnosis); and whole blood, buffy coat, and serum (VL, CL, and ML diagnosis). Samples should be taken in duplicate. |
| Sample transport | Depending on sample material, samples have to be used fresh or can be collected in tubes, on filter paper, or slides, sometimes requiring the addition of reagents (e.g., buffers) to stabilize the materials. If not used fresh, samples collected in the field have to be stored accordingly, usually on ice and then at 4°C or $-20$°C; long-term storage should be done at $-40$°C and below. |
| Pretreatment of samples | Prior to DNA extraction, several methods are available to increase DNA yield and the probability of detecting pathogen DNA in a clinical sample, including sonication, incubation with proteinases, and lysis using a range of buffers. |
| DNA extraction | Standard DNA extraction protocols include the use of phenol-chloroform, Chelex resin, or silica. Several commercial DNA kits have been used successfully to extract *Leishmania* DNA from clinical samples. Extracted DNA may have to be diluted in water prior to amplification in order to prevent PCR inhibition. |
| Choice of PCR primers and platform | Over the years, several PCR primers have become available and target either the kinetoplast or rRNA gene, internal transcribed spacers, mini-exon genes, and specific gene sequences (e.g., glycoproteins, heat shock proteins, and cysteine proteinases); primers can be either genus, subgenus, or species specific. A range of PCR platforms and approaches exist (as described in the text). |
| Additional steps | Once amplified, the conventional approach has been to visualize PCR amplification products on ethidium bromide-stained agarose gels under UV light. Platforms that do not require that step are becoming increasingly used, e.g., real-time PCR (7) or oligochromatographic PCR (5). The sensitivity and specificity of most protocols can be significantly increased by hybridization to genus- or species-specific probes. Originally, these probes were labeled with radioactive isotopes, but they are now commonly labeled with fluorescent dyes. The specificity of the protocols can also be increased by cleaving PCR amplification products with restriction enzymes, yielding species- and strain-specific restriction patterns that can be visualized after electrophoresis. |
| Standardization | All PCR assays should have, at least, DNA extraction controls (i.e., naive samples spiked with known amounts of pathogen DNA, as well as a sample with water) (21), internal amplification controls (i.e., host DNA), negative and positive in-run controls; replicate assays should be carried out. Laboratories should also allow for a mechanism of external quality control. |

gene amplification (8). Comparison between studies should be done with extreme care, taking into consideration (i) the clinical context of the study and (ii) the clinical and laboratory criteria used for defining cases and noncases. In the absence of a real gold standard for diagnosis of leishmaniasis, this may have important consequences for the definitions of sensitivity and specificity. Comparative studies of protocols should be encouraged and updated to the latest technological developments in the field and, most importantly, should be done as multicenter studies with adequate sample sizes to allow for statistical comparisons of evaluated diagnostic protocols. Samples and protocols should be exchanged, same case definitions for determination of sensitivity and specificity should be used, protocol of cost-effectiveness should be determined, and standard operating procedures should be recommended (see the report of an expert group [http://www.leishmed.net]).

## CONCLUSION AND FUTURE PERSPECTIVES

PCR-based protocols have increased the speed and sensitivity of species-specific leishmaniasis diagnosis compared to the conventional techniques such as microscopy and parasite culture. However, PCR-based protocols urgently need standardization and optimization. Recommendations include usage of extraction controls, internal controls, a *Leishmania* standard control, replicate assays, and participation in an external quality control program. Compared to other diagnostic techniques available, the molecular approaches remain expensive and require technological expertise, and efforts should be made to make PCR platforms more user-friendly and cost-effective, especially in remote areas where leishmaniasis is endemic.

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