Short Report: *Leishmania major*, the Predominant *Leishmania* Species Responsible for Cutaneous Leishmaniasis in Mali

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Abstract. *Leishmania major* is the only species of *Leishmania* known to cause cutaneous leishmaniasis (CL) in Mali. We amplified *Leishmania* DNA stored on archived Giemsa-stained dermal scraping slides obtained from self-referral patients with clinically suspected CL seen in the Center National d'Appui A La Lutte Contre La Maladie (CNAM) in Bamako, Mali, to determine if any other *Leishmania* species were responsible for CL in Mali and evaluate its geographic distribution. Polymerase chain reaction (PCR) amplification was performed using a *Leishmania* species-specific primer pair that can amplify DNA from *L. major*, *L. tropica*, *L. infantum*, and *L. donovani* parasites, possible causative agents of CL in Mali. *L. major* was the only species detected in 41 microscopically confirmed cases of CL from five regions of Mali (Kayes, Koulikoro, Ségou, Mopti, and Tombouctou). These results implicate *L. major* as the predominant, possibly exclusive species responsible for CL in Mali.

Cutaneous leishmaniasis (CL) is a skin infection caused by the hemoflagellate *Leishmania*, which is transmitted by the sand fly bite. CL lesions are characterized by an ulcer with indurated borders and a necrotic base that often heals without treatment months after the initial infection. The geographic distribution of CL is quite varied and found in 88 countries on five continents, with 1.5–2.0 million new cases reported yearly worldwide. CL is often divided into Old World (including southern Europe, the Middle East, parts of southwest Asia, and Africa) and New World (from southern United States through Latin America to South America) forms depending on the geographic setting of the infection. Approximately 20 different *Leishmania* species cause CL, although the species responsible for New World and Old World CL are distinct. For example, *L. tropica*, *L. major*, *L. aethiopica*, *L. infantum*, and *L. donovani* parasite species have been shown to cause Old World CL.¹

Epidemiological studies of CL using the leishmanin skin test have shown that CL is widely distributed throughout Mali, particularly in the northern Sahelian areas.^{2–6} *L. major* is the only Old World *Leishmania* species detected in Mali. Before 2009, *L. major* had been isolated from two skin lesions, one from a visitor to Mali and the other from a Malian.^{7,8} A more recent study identified four different *L. major* strains in Mali.⁹ Although these reports established the presence of *L. major* in the country, the frequency of specific etiologic agents and the extent of their geographical distribution in Mali remain unknown. In this study, we used *Leishmania* species-specific polymerase chain reaction (PCR) to confirm the presence and distribution of *L. major* and determine if other *Leishmania* species exist or are clinically relevant in Mali. We show that *L. major* is the predominant and probably exclusive species of *Leishmania* found in Mali. Moreover, *L. major* was found in various geographic and ecoclimatic zones across the country.

Eighty-five archived anonymized Giemsa-stained skin scrapings of individuals with microscopically confirmed CL who presented to the Center National d'Appui A La Lutte Contre La Maladie (CNAM) in Bamako, Mali between January of 2005 and March of 2006 were used in this study (National Institutes of Health Institutional Review Board exemption #11605). As part of the National Ministry of Health, CNAM is the only referral clinic for skin diseases in Mali and receives patients from the entire country. Parasite DNA extraction was performed using the techniques described elsewhere.^{10,11} Briefly, Giemsa-stained specimens were rehydrated in 200 μ L lysis buffer (100 mM Tris HCl [pH 7.5], 5 mM ethylenediaminetetraacetic acid [pH 8.0], 0.2% sodium dodecyl sulfate, 200 mM NaCl, and Proteinase K freshly added at 200 μ g/mL) while on the slide. The DNA was then precipitated with isopropyl alcohol, centrifuged, and resuspended in water.

Leishmania DNA was amplified by PCR as described by Anders and others.¹² The Uni21 and Lmj4 primer pair amplifies *L. major*, *L. tropica*, *L. infantum*, and *L. donovani* DNA.¹² Amplified DNA from each *Leishmania* species differs in size, allowing for species identification based on the length of the PCR fragment.¹² For example, *L. major* DNA yields a 650-bp product, whereas *L. donovani* DNA produces an 800-bp fragment. The positive controls were DNA from cultured *L. major* and *L. donovani*. The negative control was a blank sample (water) processed in parallel with our test samples from the beginning of the DNA extraction. To control for sample DNA quality, β -actin was amplified from each sample.¹³

In addition to identifying *Leishmania* species on the basis of PCR product size, representative PCR products were sequenced for species confirmation. Because of the difficulties with direct sequencing of the PCR products using the Uni21 and Lmj4 primers, we cloned the gel-purified PCR reaction into pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The clones were sequenced directly using M13 forward and reverse primer pair. The resulting sequence was analyzed using DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI). Sequences were compared with published sequences from kinetoplast DNA

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FIGURE 1. *L. major* is the only parasite species identified by PCR in clinically suspected cases of CL in Mali. Positive control lanes + *L. major* and + *L. donovani* contain 650-bp and 800-bp PCR products from samples containing *L. major* and *L. donovani* DNA. Negative control lane (NC) shows PCR result from a sample processed in parallel to DNA extracted samples. Lanes 1–4 contain PCR products from four microscopically suspected samples of CL. M lane contains 100-bp DNA ladder.

(kDNA) from *L. major* (GenBank accession no. J04654), *L. infantum* (GenBank accession no. AF188701), *L. tropica* (GenBank accession no. Z32841), and *L. donovani* (GenBank accession no. AF167718).

Of 85 archived Giemsa-stained, microscopically confirmed slides, 41 (48%) were found to contain *Leishmania* DNA by PCR. All 41 PCR products were approximately 650 bp in size, comparable with the *L. major* DNA control and clearly different in size than the *L. donovani* DNA control and expected sizes for the other *Leishmania* species (specifically *L. infantum* and *L. tropica*, which are indistinguishable from *L. donovani* using this primer set), indicating that these patients were infected with *L. major* (Figure 1). To confirm the presence of *L. major* DNA, four randomly selected samples were sequenced. Based on PCR product size and sequence confirmation, these findings suggest that *L. major* is the predominant, if not the exclusive, species responsible for CL in Mali.

PCR-confirmed samples came from various geographic and ecoclimatic zones across the country (north Sudan savanna, Sahelian areas, and sub-Saharan zones) (Figure 2), with the exception of the southern part of the country (south Sudan savanna). The majority came from the Sahelian areas and North Savanna areas. Figure 2 does not suggest that CL is found only in the locations indicated, because regions farther away from the Koulikorou region (the location of CNAM) may not be adequately represented because of the self-reporting nature of CL in Mali.

Species identification is important for characterizing the epidemiological and clinical spectrum of CL in Mali. As confirmed by our study of archived samples, CL is endemic in several regions of Mali; however, the disease is underreported and unknown to medical personnel in remote areas. Typically, only patients with chronic and persistent CL are referred to the CNAM dermatology clinic after treatment failure or as a result of misdiagnosis. Thus, CL cases received from this

FIGURE 2. Geographic distribution of PCR-confirmed cases of CL in Mali. Color coding represents frequency of cases originating from each district.

clinic provide a good representative sample of the geographic distribution of chronic CL in Mali. Species identified in these patients may also be a good representative sample of the parasite population responsible for the disease.

We used PCR to test for the presence of four species (*L. major*, *L. tropica*, *L. infantum*, and *L. donovani*) potentially responsible for CL in Mali. Of the 85 slides identified as positive by microscopy, we confirmed the presence of *Leishmania* DNA in 41 archived samples. The presence and integrity of DNA in the *Leishmania* PCR-negative samples were established by PCR using a housekeeping gene, β -actin (data not shown). At the onset of this project, DNA was extracted from only one of three skin scrapings on each Giemsa-stained slide. Despite the possibility that not all specimens on each slide contained amastigotes, we decided to pursue this study design, because our objective was not to confirm clinically and microscopically suspected cases but identify the diversity of *Leishmania* species; PCR confirmation of over 40 specimens was sufficient for this purpose. In addition to the study design, it is possible that PCR, at least in the context of our protocol, is not as sensitive as Giemsa for the detection of *Leishmania* DNA. Whereas the presence of a single amastigote is sufficient to deem a specimen positive by microscopy, it is possible that many more parasites are needed for detection with our PCR method. This finding may be especially true in cases of chronic CL, where the parasite burden per lesion is much lower. The primer pair used in this study has been shown to reliably detect numerous *Leishmania* species.¹² We tested the primer pair on *L. major* and *L. donovani* DNA and showed that we were able to amplify DNA fragments from these two *Leishmania* species. Therefore, it is unlikely that our primer pair selectively detected *L. major* in our microscopy-confirmed cases.

Using the kDNA PCR, one of the most sensitive PCR methods to detect *Leishmania* DNA,^{12,14} we found that *L. major* is the only species present in archived specimens from individuals diagnosed with CL at CNAM. The dermatology clinic of CNAM is the only national institution specializing in the treatment of CL and other skin diseases in Mali. Access to CNAM is difficult for much of the population in Mali, specifically from the northern regions. Thus, other *Leishmania* species may exist in Mali, but they seem to be rarely observed in the geographic areas where *L. major* was found. Along with three previous reports confirming the presence of *L. major* in Mali,⁷⁻⁹ this study suggests that *L. major* is likely the sole species responsible for CL in Mali and that it is found throughout the country.

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