Diagnostic Efficacy of Molecular Techniques for Detection and Identification of *Leishmania* Species in Human Whole Blood and Skin Samples from Ecuador

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Abstract. Microscopic examination is the standard method for diagnosis of cutaneous and mucocutaneous leishmaniasis despite its low sensitivity. This study compared the diagnosis efficacy of microscopic examination versus polymerase chain reaction (PCR)-based methods and DNA sequencing using whole blood and skin lesion samples from patients with suspected leishmaniasis. The presence of *Leishmania* was determined by microscopy and amplification of 18S ribosomal RNA gene from blood and skin samples of 22 patients. Twenty individuals were positive for leishmaniasis. Microscopic analysis identified 85%, whereas PCR identified 100% of positive cases from skin and 90% from blood. *Cytochrome b* gene (*cyt-b*) amplification and sequencing identified *Leishmania guyanensis*, *Leishmania shawi*, and *Leishmania naiffi* from skin and blood samples. This study demonstrated the usefulness of whole blood and molecular techniques for the diagnosis and species identification of leishmaniasis.

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the *Leishmania* genus.¹ Leishmaniasis has become an important public health problem in tropical and subtropical regions of the world.² In Ecuador, leishmaniasis infection has been reported in 23 of the 24 provinces (excluding only the Galápagos islands).³ The clinical presentation and disease progression of leishmaniasis depend on the causative species and the immune status of the patient.⁴ In Ecuador, human cutaneous and mucocutaneous leishmaniasis (CL and MCL, respectively) and their variations have been confirmed.³ CL is a common form of the disease characterized by the appearance of a localized skin lesion at the site of the vector-bitten area.⁵ In 5–20% of CL, the infection progresses to MCL, due to the dissemination of the parasite in the bloodstream, the lymphatic system, or by direct extension to mucosal membranes.⁶ A total of 15 species of *Leishmania* have been identified as responsible for the infection of humans.² In Ecuador, only *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania major*-like, *Leishmania mexicana*, *Leishmania panamensis*, and *Leishmania naiffi* have been identified.^{7,8}

Traditionally, microscopic examination has been considered the standard method for diagnosis of CL and MCL. However, despite its high specificity (100%),⁹ its sensitivity is low (49–74.4%).¹⁰ More importantly, microscopy cannot identify specific *Leishmania* species causing the infection.⁹ More sensitive polymerase chain reaction (PCR)-based methods have been developed as an alternative for diagnosis and identification of *Leishmania* species in clinical samples.¹⁰ PCR-based diagnosis has been used with cutaneous lesions and isolated white blood cells,^{11,12} but not with whole blood.

Given the limitations of standard methods for the diagnosis and identification of *Leishmania* species in clinical samples, we compared the diagnostic efficacy of traditional microscopy to PCR-based methods and DNA sequencing using whole blood and skin samples from patients with

suspected leishmaniasis. Twenty-two patients with clinical suspicion of CL were included in this study. Patients were recruited at a public clinic within a rural area endemic for leishmaniasis in “Pedro Vicente Maldonado” (0°08’31”, –79°05’33”) and a military reference hospital “Hospital de Especialidades de las Fuerzas Armadas No. 1” in Quito. All participants were tested by microscopy and molecular techniques. Samples were collected from cutaneous lesions according to the specimen collection guide for cutaneous leishmaniasis.¹³ In addition, 3 mL of venous blood was collected in tubes with ethylenediaminetetraacetic acid (Becton Dickinson, Franklin Lakes, NJ). Cutaneous and blood samples were stored at 4°C and processed within 24 hours of collection. DNA from cutaneous lesions and blood was isolated with the High Pure PCR Template Preparation Kit (Roche®, Quito, Ecuador). To diagnose leishmaniasis infection, PCR amplification of the 18S ribosomal RNA (rRNA) gene was performed as reported previously.¹⁴

Of the 22 recruited patients, only 10 had not received treatment of leishmaniasis at the time of sample collection. Table 1 compares the results of diagnosis for leishmaniasis by microscopy and by PCR amplification of the 18S rRNA gene from skin lesions. Data showed that seventeen skin lesions were positive for leishmaniasis by microscopy and PCR analysis. On the other hand, three skin lesions were positive by PCR and negative by microscopy. Finally, two skin samples were negative for both PCR and microscopy; these two patients were not under antileishmaniasis treatment at the time of recruitment. According to this table and considering microscopy as the gold standard, the sensitivity of PCR of skin lesions was 100% (17 of 17) and the specificity was 40% (two of five).

Subsequently, we wanted to compare the efficacy of diagnosis between PCR analysis from two different biological samples, skin and whole blood, since blood is not routinely used for CL diagnosis. Data showed that PCR analysis of skin samples identified 20 positive cases, whereas blood PCR identified 18, Figure 1A. Skin and blood samples from two patients were negative for leishmaniasis by PCR. We also evaluated the agreement in the diagnosis of leishmaniasis between microscopy and blood PCR analysis. Data showed that 16 of the 17 patients that had a diagnosis of leishmaniasis

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TABLE 1

Diagnostic efficacy of skin and whole blood PCR compared with microscopy for the diagnosis of cutaneous leishmaniasis

		Microscopy		Total	Sensitivity	Specificity
		+	-			
Skin lesion PCR	+	17	3	22	17/17 = 100%	2/5 = 40%
	-	0	2			
Whole blood PCR	+	16	2	22	16/17 = 94.1%	3/5 = 60%
	-	1	3			

PCR = polymerase chain reaction.

by microscopy were positive by blood PCR (94.1%); on the other hand, of the 18 blood samples that were positive for leishmaniasis by blood PCR, 16 were positive by microscopy (88.9%); three patients were negative for leishmaniasis by blood PCR and microscopy. These data indicated good concordance between blood PCR and microscopic analysis for the diagnosis of leishmaniasis, 19/22 (86.4%). According to these results and considering microscopy as the gold standard, the sensitivity of PCR for whole blood was 94.1% (16 of 17) and the specificity was 60% (three of five), Table 1.

Previous studies indicate that variations on *cyt-b* gene distinguish *Leishmania* species.^{15,16} We amplified the *cyt-b* gene in skin and whole blood samples that were positive for 18S rRNA *Leishmania* gene ($N = 20$) as reported previously.¹⁵ Eight samples that were positive for 18S rRNA also amplified for the *cyt-b* gene; four from skin and four from blood, Figure 1B. To identify *Leishmania* species, quantitative PCR products were purified (Beckman Coulter, Pasadena, CA) and subsequently sequenced in a 3500 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA). Data were analyzed (SeqScape® v2.5 software, Foster City, CA) and the nucleotide sequences of the *cyt-b* gene for the various *Leishmania* species, available in the GenBank, were compared through sequence alignments and phylogenetic trees.^{15,16} In three different patients, we were able to identify species-specific sequences from the eight sequenced samples; *L. guyanensis* and *Leishmania shawi* were identified from two cutaneous samples, whereas *L. naiffi* was detected in blood.

The identification of *Leishmania* species in patients with CL has important implications related with the natural course of infection, its treatment, and the epidemiology of the disease.¹⁷ For instance, *Leishmania tropica*, *L. major*,

Leishmania aethiopica, and *L. mexicana* complexes cause CL¹⁸; disseminated cutaneous leishmaniasis is caused by *L. braziliensis*, *L. amazonensis*, and *L. guyanensis*¹⁹; and MCL is usually caused by *L. braziliensis*¹⁹ and in rare cases by *L. panamensis* and *L. guyanensis*.¹⁷ *Leishmania* species also respond differently to treatment,^{17,18,20} for example, species-specific antimonial sensitivity has been determined in an in vitro study, in which *L. tropica*, *L. braziliensis*, and *L. panamensis* were 3, 2.3, and 6 times more resistant than *L. major* to this drug.²⁰ Identification of *Leishmania* species is critical for the diagnosis and treatment of this infection. Use of present PCR diagnosis techniques will contribute to improve the clinical management of this parasitic disease as well as to better understand the anthroponotic transmission of the infection.¹¹

Previous studies have used blood samples to identify *Leishmania* parasites by PCR.^{11,12,21} Venazzi and others,¹² studying 68 patients with CL, detected parasite DNA in 3.4% of prepared blood samples. In that study, blood samples were treated with a solution containing 25 mM citric acid, 50 mM sodium citrate, and 81 mM glucose before DNA isolation. Vergel and others¹¹ detected the presence of *Leishmania* DNA in 7.5% of skin lesions and in 19.2% in peripheral-blood monocytes isolated from 59 patients with confirmed diagnosis of CL. In a recent study, Neitzke-Abreu and others²¹ reported the identification of *Leishmania* DNA in 83.87% of skin lesions and 51.67% of peripheral-blood monocytes isolated from 106 patients with CL. The percentages of *Leishmania* DNA detected in the studies described above are lower than the percentage of *Leishmania* DNA detected in the skin and whole blood of the present study. It is possible that the observed differences for the detection of parasite DNA could be due to the methods used in previous studies to prepare the blood or to isolate the peripheral-blood monocytes. Herein, we did not manipulate the blood samples, and DNA was directly isolated from samples within 24 hours of collection. These data indicate that it is not necessary to either treat blood samples or isolate white blood cells from the samples of *Leishmania*-infected patients to be able to detect the parasite DNA. Rather, direct DNA isolation from whole blood could be a better source of DNA for leishmaniasis diagnosis. It is important to note that we were able to detect parasite DNA even in patients who had already started the conventional treatment with meglumine antimoniate.

FIGURE 1. Presence of (A) 18S rRNA gene and (B) *cyt b* gene in skin and whole blood samples of patients with suspected cutaneous leishmaniasis. In (A), lane 1 = 100-base pair (bp) molecular marker; lane 2 = positive control (*Leishmania equatorensis* DNA); lines 3–5 = skin (s) and blood (b) samples; lane 6 = negative control. In (B), lane 1 = 100-bp molecular marker; lines 2–8 = skin (s) and blood (b) samples; lane 9 = negative control; lane 10 = positive control (*L. equatorensis* DNA). Number indicates the number of the patient in the study.

Highest diagnostic sensitivity was obtained by 18s rRNA gene PCR analysis. Consequently, a potential gold standard for CL should be based on molecular analysis rather than microscopic examination. PCR analysis can also be successfully performed in whole blood samples from patients suspected of CL and MCL. Blood samples are more easily obtained and require less-invasive methods than skin scraping from suspected patients.

We acknowledge that there were limitations in the identification of specific *Leishmania* species for all the positive samples since *cyt-b* gene was amplified in eight of the 20 positive samples and in three of the samples in which the species of *Leishmania* were identified. It is possible that the number of copies of the gene in the clinical samples were not at the level of detection for PCR amplification. Since most patients were under treatment of leishmaniasis at the time of recruitment, it is likely that the parasitic load was decreased at the time of specimen collection. It is also possible that difficulties of the processing and purification of *cyt-b*-amplified products could have affected sample sequencing.

We conclude that it is critical to establish a simple and well-defined protocol for molecular analysis, not only for *Leishmania* detection, but also for species identification. The current study showed the feasibility and high efficacy of leishmaniasis diagnosis by the identification of the species in whole blood and skin samples using PCR amplification and sequencing. This study also reports for the first time the presence of *L. shawi* in Ecuador.

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