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Prevalence of and Factors Associated with Negative Microscopic Diagnosis of Cutaneous Leishmaniasis in Rural Peru

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Abstract. Cutaneous leishmaniasis is endemic to South America where diagnosis is most commonly conducted via microscopy. Patients with suspected leishmaniasis were referred for enrollment by the Ministry of Health (MoH) in Lima, Iquitos, Puerto Maldonado, and several rural areas of Peru. A 43-question survey requesting age, gender, occupation, characterization of the lesion(s), history of leishmaniasis, and insect-deterrent behaviors was administered. Polymerase chain reaction (PCR) was conducted on lesion materials at the Naval Medical Research Unit No. 6 in Lima, and the results were compared with those obtained by the MoH using microscopy. Factors associated with negative microscopy and positive PCR results were identified using χ^2 test, *t*-test, and multivariate logistic regression analyses. Negative microscopy with positive PCR occurred in 31% (123/403) of the 403 cases. After adjusting for confounders, binary multivariate logistic regression analyses revealed that negative microscopy with positive PCR was associated with patients who were male (adjusted odds ratio [OR] = 1.93 [1.06–3.53], *P* = 0.032), had previous leishmaniasis (adjusted OR = 2.93 [1.65–5.22], *P* < 0.0001), had larger lesions (adjusted OR = 1.02 [1.003–1.03], *P* = 0.016), and/or had a longer duration between lesion appearance and PCR testing (adjusted OR = 1.12 [1.02–1.22], *P* = 0.017). Future research should focus on further exploration of these underlying variables, discovery of other factors that may be associated with negative microscopy diagnosis, and the development and implementation of improved testing in endemic regions.

INTRODUCTION

Leishmaniasis describes a disease caused by infection with a protozoan parasite in the genus *Leishmania*, which can manifest as three forms: cutaneous, mucocutaneous, and visceral. Leishmaniasis is transmitted to the human host by the bite of infected phlebotomine sand flies of the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World.^{1–3} This disease is estimated to cause around 51,000 deaths worldwide each year, with global incidence rising annually.⁴ Currently, the most common form of leishmaniasis is cutaneous, with close to 1 million reported cases in the past 5 years.⁵ Cutaneous leishmaniasis (CL) occurs in the Americas, the Mediterranean Basin, and western Asia (from the Middle East to Central Asia) with 70–75% of cases occurring in 10 countries: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru.⁶

In developing countries, routine diagnosis of all forms of leishmaniasis often combines clinical symptoms with direct microscopic examination, serology, and culture. However, clinical manifestations are often varied and symptoms can be sub-clinical, whereas culture methods are time-consuming and often produce no yield.^{7,8} All of these factors contribute to the low sensitivity associated with these methods.⁹ On the other hand, molecular methods, such as polymerase chain reaction (PCR), are used primarily in research and approach 99–100% in both sensitivity and specificity.^{10–15} Although direct visualization of the parasite is the most commonly used technique and often considered the gold standard of diagnosis for both visceral leishmaniasis (VL) and CL, there is currently no consensus on which method should be regularly used.^{16,17}

In most rural clinical laboratories, microscopy is used for routine diagnosis, creating an opportunity for inaccurate diagnoses, which can impact the patient and the health-care system.⁸ False positives may lead to unnecessary therapies with potentially toxic drugs, whereas false negatives can result in prolonged disease courses, increased resource utilization for repeat testing, and greater patient suffering.¹⁸ For example, it has been established that reinfection and drug resistance can occur if treatment of leishmaniasis is not appropriate or timely.¹⁹

Although studies have begun to explore risk factors associated with false-negative PCR results in the diagnosis of leishmaniasis, few data describe the risk factors for negative microscopy/positive PCR results, especially in endemic settings.²⁰ This study seeks to elucidate any relationship between demographics, lesion characteristics, and/or insect deterrent behaviors and negative microscopy/positive PCR results. This information could prove vital in identifying individuals at risk for false-negative diagnosis, who could benefit from alternative diagnostic methods or further workup in leishmaniasis-endemic areas where microscopy is routinely used for diagnosis.

MATERIALS AND METHODS

Ethics. This study was approved by the Naval Medical Research Unit No. 6 (NAMRU-6) Institutional Review Board. Adult participants provided written informed consent and participants aged 8–17 years provided written informed assent. Parents of participants aged 5–17 years provided written informed parental consent.

Study design. The parent study of this project was a cross-sectional, sentinel surveillance study conducted from 2007 to 2015, which enrolled 485 patients aged 5–65 years at the following study sites in Peru: Lima, Iquitos, Puerto Maldonado, as well as other smaller and more remote locations (Figure 1).

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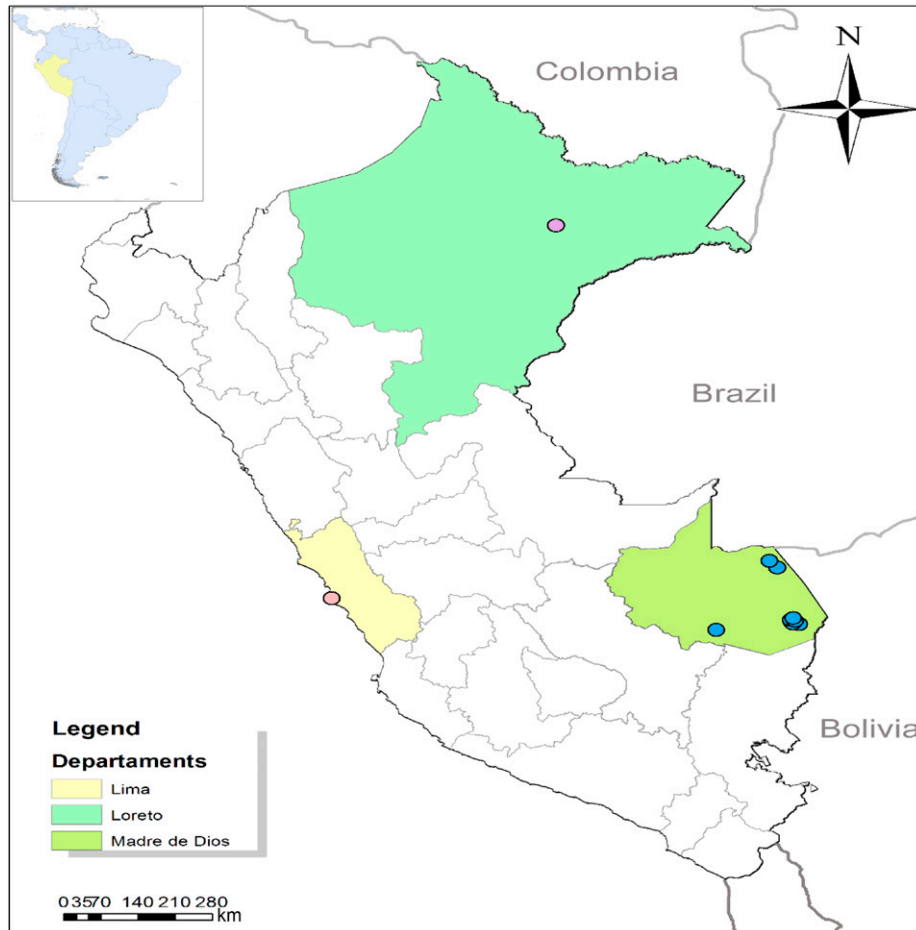


FIGURE 1. Map of lesion collection sites included in this study. This figure appears in color at www.ajtmh.org.

Individuals who were identified with suspected or confirmed CL were referred by Ministry of Health (MoH) staff to one of the study sites. The study described here is a nested, case-control study, using 403 eligible cases from the parent study. Eighty-two cases from the parent study were deemed ineligible and eliminated from the present study because they were identified in the military hospital in Lima and did not have microscopy data to use in the analysis.

Inclusion/exclusion criteria. *Inclusion criteria.* The inclusion criteria were as follows: 1) participant aged 5–65 years, 2) laboratory-confirmed case or clinical suspicion of CL (clinical suspicion consisted of the presence of an erythematous papule, nodule, plaque, ulcer, or scar at the site of a suspected sand fly bite, with or without raised and reddened lesion borders, ulcers that were large and painless, unless secondary infection had occurred, and/or lesions associated with lymphadenopathy), and 3) signed informed consent/assent.

Exclusion criteria. The exclusion criteria were as follows: 1) currently under treatment of leishmaniasis or 2) had undergone treatment of leishmaniasis in the past 6 months.

Specimen collection. After explaining the study procedures, risks, and benefits and obtaining informed consent/assent, a trained MoH health-care worker and/or NAMRU-6 researcher collected the most appropriate clinical samples as described in the following paragraphs. Preliminary diagnosis was performed on-site by direct light microscopy examination. Further confirmation of diagnosis and species via PCR

was performed by the NAMRU-6 in Lima. Different collection methods were used to gather as much clinical data as possible. When applicable, all methods were used to obtain the samples.

Filter paper sampling. After cleaning the affected skin area with sterile gauze and alcohol, a 9-cm Fisher-brand, wet-strengthened qualitative filter paper (Fisher Scientific, Pittsburgh, PA) was gently pressed on the ulcer base. The filter paper was allowed to air-dry. Then, 10-mm filter-paper punches were stored in 1.5-mL microcentrifuge tubes containing 100% ethanol.

Lancet sampling. A cotton swab with 70% ethanol was used to clean around the lesion border. Using a new, Goldenrod animal lancet, exudate from the inner margin of the lesion border was obtained. With this exudate, two slides were made for microscopy diagnosis on-site at the study locations. Using a second, new lancet, more exudate from inside the border region of the lesion was obtained and placed in a microcentrifuge tube (1.5 mL) containing 1 mL of 70% ethanol. With this exudate, additional two slides were made for an archive and confirmatory diagnosis at NAMRU-6, Lima.

Biopsy sampling. Local anesthetic (i.e., Xylocaine 5%) was injected at the border of the clean lesion, and a 3.5–4 mm diameter punch biopsy was obtained from the lesion border. The biopsy sample was split into 2–3 parts, depending on the size, and placed in 1.5 mL microfuge tubes containing 1 mL of 70% ethanol. All samples were stored for 1–2 weeks at research sites before being transported to NAMRU-6 facilities

in Lima. Aliquots from these specimens were used for DNA isolation and PCR analysis for leishmaniasis diagnosis.

Patient questionnaire. A 43-item questionnaire was administered to each consenting study participant or their legal guardian to obtain information regarding age, gender, occupation, location, and characterization of the lesion(s), history of leishmaniasis, insect-deterrent behaviors, and other factors (Supplemental Questionnaire).

Data analysis. Polymerase chain reaction analysis targeting kDNA was conducted on lesion materials obtained from filter paper samples, lancet-extracted exudate, and punch biopsies following protocols described in the literature.²¹ If a positive PCR result was obtained from one or more of the specimens collected from a patient, then he/she was considered positive by PCR. Based on the questionnaire, a preliminary analysis of 131 cases from the research sites in Madre de Dios was performed. Risk factors found to be associated with negative microscopy/positive PCR via χ^2 test and *t*-tests during the initial analysis were included in the present study's investigation. The included risk factors were examined using cross-tabular analyses with χ^2 of independence when data were categorical and independent two-sample *t*-tests when data were continuous (nonparametric tests were also conducted on these data, and findings were identical). The factors included in the present analysis were age, gender, occupation, number of lesions, diameter of the lesion, previous leishmaniasis, time from appearance of the lesion to PCR-based diagnosis, presence of concurrent mucosal leishmaniasis, presence of superimposed bacterial infection of the lesion, and whether the patient's residence had been fumigated within the last 6 months.

Of note, for the analysis of occupation, categories included agricultural workers/wood extractors and gold extractors (both classified as high-exposure-to-sand fly groups because they work outdoors), students (low-exposure group), and others. The agricultural worker/wood extractor occupation group was chosen as the reference group based on the hypothesis that this group likely had the highest exposure to sand flies because workers in these occupations directly encroach on forested sand fly habitats.¹⁹

Binary multivariate logistic regression modeling was used to estimate the adjusted odds of a false-negative microscopy reading for identified risk factors, with 95% confidence intervals also being calculated. By convention, all tests of significance were two-tailed and evaluated at the level of $P < 0.05$. Data analyses were conducted using SAS 9.3 (SAS Institute Inc., Cary, NC).

Eighty-two cases were not eligible for the study because these patients presented to Hospital Militar Central in Lima and microscopy data were not readily available to use in the analysis. Cross-tabular analyses with χ^2 test of independence and independent two-sample *t*-tests were also conducted to elucidate any differences between the eligible and ineligible groups.

RESULTS

Among the 485 individuals in the parent study who tested positive for leishmaniasis by PCR, microscopy was not performed on the 82 participants who were excluded from the nested, case-control study. Demographic, location of enrollment, and occupation data were collected and recorded (Table 1). Of the 403 eligible individuals with PCR positive

diagnosis, microscopy yielded 123 negative and 280 positive results. Using the PCR-obtained results for comparison, microscopy was 56% (157/280) sensitive and 94% (116/123) specific for diagnosing leishmaniasis (Table 2).

Patients with negative microscopy/positive PCR results were first compared with patients with all other results using χ^2 test and *t*-tests. Patients with negative microscopy/positive PCR were more likely to have larger lesion diameters (24.7 versus 19.9 mm, $P = 0.010$), multiple lesions (40% versus 28%, $P = 0.036$), previous history of leishmaniasis (46.2% versus 26.8%, $P = 0.001$), and longer duration between lesion appearance and PCR testing (2.90 versus 2.07 months, $P = 0.003$). Finally, negative microscopy/positive PCR tended to occur in younger patients (27.0 versus 29.9 years, $P = 0.069$), although this difference was not statistically significant (Table 3). Negative microscopy/positive PCR results were not significantly associated with gender, occupation, concurrent mucosal leishmaniasis, concurrent bacterial infection of the lesion, or fumigation of the participant's residence in the preceding 6 months.

Binary multivariate logistic regression analyses demonstrated that male patients were almost twice as likely to have negative microscopy/positive PCR (adjusted OR = 1.93 [1.06–3.53], $P = 0.032$), patients who had previous leishmaniasis were around three times as likely (adjusted OR = 2.93 [1.65–5.22], $P < 0.0001$), and patients who had larger lesions (adjusted OR = 1.02 [1.003–1.03], $P = 0.016$) and/or had a longer duration between lesion appearance and PCR testing (adjusted OR = 1.12 [1.02–1.22], $P = 0.017$) were also at increased risk for negative microscopy/positive PCR results (Table 4). Again younger age was seen as marginally statistically related to higher negative microscopy/positive PCR rates (adjusted OR = 0.98 [0.96–1.002] $P = 0.07$). Factors that were

TABLE 1
Demographic, location of enrollment, and occupation information for the 403 cases included in the study

Demographic information	Total
Age (years)	
≥ 60	17
50–59	18
40–49	41
30–39	79
20–29	136
10–19	91
0–9	18
N/A	3
Gender	
Male	317
Female	86
Location of enrollment	
Hospital Militar Central, Lima	2
Hospital Iquitos, Iquitos	55
Puerto Maldonado (Centro de Salud Jorge Chavez, Hospital Santa Rosa, Centro de Salud Nuevo Millenio, Laboratorio Referencial, Hospital Tambopata, Puesto de Salud Flor de Acre), Madre de Dios	258
Delta 1, Madre de Dios	74
Hospital Iberia, Madre de Dios	13
Puesto de Salud Otilin, Madre de Dios	1
Occupation	
Agriculture/wood extraction	87
Gold extraction	59
Student	96
Other	161

TABLE 2

Results of microscopy and PCR analysis for the 403 cases included in this study

		PCR results		Total
		+	-	
Microscopy results	+	157	7	164
	-	123	116	239
	Total	280	123	403

PCR = polymerase chain reaction.

not significantly associated after adjustment included occupation, number of leishmaniasis lesions, concurrent mucosal leishmaniasis, concurrent bacterial infection of the lesion, and fumigation of the participant's residence in the preceding 6 months.

Finally, analyses performed on the 82 ineligible cases found that compared with the 403 cases eligible for this study, a higher proportion were male, identified as students or other for their occupation, and had a concurrent bacterial infection. Besides gender, there were no significant differences between those eligible and ineligible regarding any of the variables that were found to be associated with negative microscopy/positive PCR results in this study.

DISCUSSION

Our study found that compared with PCR results, microscopy has a sensitivity of 56% in the diagnosis of leishmaniasis.

TABLE 3

Patient demographics, lesion characteristics, and proposed risk factors for participants with negative microscopy/positive PCR as compared with patients with all other results

	False negative (N = 123)	Other (N = 280)	P value
Age in years, mean (SD)	27.0 (13.3)	29.9 (14.8)	0.069
Gender			
Male, no. (%)	91 (28.7)	226 (71.3)	0.129
Female, no. (%)	32 (37.2)	54 (62.8)	
Occupation			
Agriculture/wood extraction, no. (%)	28 (32.2)	59 (67.8)	0.402
Gold extraction, no. (%)	15 (25.4)	44 (74.6)	
Student, no. (%)	35 (36.5)	61 (63.5)	
Others, no. (%)	45 (28)	116 (72)	
Number of lesions			
One, no. (%)	86 (28)	221 (72)	0.036
Two or more, no. (%)	36 (39.6)	55 (60.4)	
Diameter of lesion in mm, mean (SD)	24.7 (19.7)	19.9 (15.2)	0.01
Previous leishmaniasis?			
Yes, no. (%)	36 (46.2)	42 (53.8)	0.001
No, no. (%)	87 (26.8)	238 (73.2)	
Duration from lesion appearance to PCR testing in months, mean (SD)	2.90 (3.05)	2.07 (2.37)	0.003
Concurrent mucosal leishmaniasis?			
Yes, no. (%)	12 (35.3)	22 (64.7)	0.528
No, no. (%)	111 (30.1)	258 (69.9)	
Concurrent bacterial infection?			
Yes, no. (%)	64 (32.5)	133 (67.5)	0.402
No, no. (%)	59 (28.6)	147 (71.4)	
Residence fumigated in past 6 months?			
Yes, no. (%)	54 (29.8)	127 (70.2)	0.939
No, no. (%)	64 (30.2)	148 (69.8)	

No. = number; PCR = polymerase chain reaction; SD = standard deviation. For categorical data, cross-tabular analyses with χ^2 test of independence were used and for continuous data, independent two-sample *t* tests were used.

Leishmaniasis diagnosis is largely based on clinical criteria with microscopy and visualization being the most commonly used method of diagnostic detection in rural areas.⁸ Complicating the situation is the variability of clinical manifestations of leishmaniasis, rendering clinical diagnosis difficult.²² In addition, patients in these areas often need a positive diagnosis to receive aid to pay for treatment. Given these considerations, any flaws in the diagnostic method are worthy of exploration, especially because undiagnosed and untreated leishmaniasis can be a disfiguring disease, advance to afflict mucosal surfaces and visceral organs, and ultimately become fatal (in the case of visceral disease).²³

This study also identified statistically significant factors that placed patients at an increased risk of receiving negative microscopy/positive PCR results. Whereas certain factors, such as male gender, may be intuitive because of the fact that males more frequently are used outside and at risk for sand fly bite, other factors identified may not. The reasons these selected factors may predispose patients to negative readings may be explained by reviewing microscopic technique and the immune response to leishmaniasis infection.

To receive a positive diagnosis of leishmaniasis via microscopy, visualization of amastigotes must be achieved. However, failure to do so can simply imply low parasite load, rather than an absence of parasites altogether.²⁴ Sand fly saliva upregulates various aspects of the immune response, including complement activation, T-cell proliferation, hemopoiesis, and the activity of antigen-presenting cells.²⁵ This immune response begins to decrease parasitic load, meaning chronic leishmaniasis infections demonstrate lower parasitic loads than acute infections.²⁶

In addition, the host immune response creates the opportunity for acquired immunological resistance to develop in patients previously infected.²⁷ Therefore, when patients become reinfected, the immune response is augmented, leading to decreased parasitic load.²⁸ Moreover, multiple lesions are the result of spread or relapse of the primary infection, or a concurrent secondary leishmaniasis infection.²⁴ Both of these scenarios involve a primary immunologic response followed by either continuation or reactivation of that response which, as explained previously, leads to decreased parasitic load. Low parasitic load is also a result of duration of time, in which the immune system clears the lesion of amastigotes, resulting in negative microscopy readings the longer the patient waits to be tested.^{13,29} This evidence could explain our observation that longer duration between lesion appearance and PCR testing, previous leishmaniasis exposure, and multiple lesions predispose patients to negative microscopy readings.

Another explanation for a low parasitic yield, and subsequent negative microscopy reading, involves acquisition of the lesion material. The World Health Organization guidelines recommend that exudative material be taken from the lesion border, where parasitic load is believed to be higher.³⁰ Although this technique is widely used in field sites, more recent studies have shown that median parasite loads are significantly higher in the lesion base and center than in the lesion border.^{31,32} This discrepancy could lead to a decreased parasitic yield because most practitioners target the lesion border for sample collection. This effect could be exacerbated as distance from the lesion center increases, meaning that a larger distance between the center of the lesion and the border could result in an even smaller parasitic yield. This could

TABLE 4

Adjusted odds ratios for risk factors associated with increased negative microscopy/positive PCR results

Risk factor	OR	95% CI	P value
Age (years)	0.981	0.96–1.002	0.071
Gender (male)	1.931	1.056–3.528	0.032
Occupation			
Agriculture/wood extraction		Reference occupation	
Gold extraction	0.905	0.392–2.091	0.816
Student	1.015	0.465–2.215	0.971
Other	0.665	0.349–1.267	0.215
Diameter of lesion	1.017	1.003–1.031	0.016
Duration from lesion appearance to PCR testing	1.116	1.020–1.221	0.017
Number of lesions (more than one)	1.545	0.894–2.672	0.119
Previous leishmaniasis (yes)	2.934	1.647–5.224	< 0.0001
Concurrent mucosal leishmaniasis (yes)	1.039	0.437–2.474	0.93
Concurrent bacterial infection (yes)	1.158	0.711–1.886	0.555
House fumigated in past 6 months (yes)	0.902	0.548–1.486	0.686

CI = confidence interval; PCR = polymerase chain reaction.

explain our observation that larger lesions predispose patients to negative microscopy results.

The finding that is perhaps most difficult to explain is the marginal inverse relationship between age and negative microscopy readings. As per the explanations given previously, one would expect that younger patients would be leishmaniasis naive and, thus, their immune response would be diminished, resulting in an increased parasitic load. However, an alternative explanation that could help explain our contradictory observation is that because adult patients are the most commonly affected population, lesions that are present in children are largely ignored or misdiagnosed. Thus, when they are finally tested, the infection could be more chronic than in adult patients.²⁸

One final hypothesis that may help explain our findings is that patients with large lesions, multiple lesions, and/or who have had the lesion for an extended period of time are more likely to have used topical substances, which has been shown to lower the sensitivity of microscopy readings.⁹

The high prevalence of negative microscopy/positive PCR readings in the diagnosis of CL demands the development of high-sensitivity, low-cost, and field-deployable diagnostic tests. Currently, there are very few alternatives for CL diagnosis under development, such as direct boil loop-mediated isothermal amplification (LAMP).³³ By contrast, a wide set of diagnostic tools have been developed for the detection of VL, such as rapid detection tests for the K39^{34,35} and K28³⁶ genes as well as LAMP tests.³⁷ It has been shown that even though all these methods are feasible in the diagnostic detection of VL, their use in the diagnosis of CL is equivocal or of no use.³⁸

The most common types of leishmaniasis in the areas included in this study are those caused by *Leishmania amazonensis*, *braziliensis*, *mexicana*, *peruviana*, *panamensis*, and *guyanensis*, which most frequently manifest as CL.^{37,39} This collection of infections, also known as New World CL, is similar in countries throughout the West and East Andes; therefore, the results of our study are largely generalizable to these regions. In addition, our results are relevant in areas afflicted with Old World CL, such as some areas of Africa

and the Middle East.⁴⁰ However, our present study may not be generalizable to areas in which the species that cause VL, diffuse, and/or mucosal leishmaniasis are predominant. Nonetheless, it is worth noting that even in these areas, leishmaniasis often begins as a cutaneous infection.⁸

We would like to acknowledge the possibility of measurement bias due to the fact that when sampling was subjected to PCR analysis, it was performed so using a combination of samples from all methods of collection (i.e., filter paper, lancet sampling, and/or punch biopsy). Lesions were called positive for leishmaniasis when one of the PCR results from the material collected was positive. However, individual results for each type of material were not recorded. Therefore, no conclusions can be made regarding the sensitivity and specificity of each individual collection method from this study.

In addition, as in most endemic areas, at each of the study sites, different personnel were responsible for obtaining lesion exudate and making the primary diagnosis for the patients. This occurred before members of this research protocol collected samples used for our analysis and may have introduced some uncontrolled variability, as those making the initial diagnosis could have had varying degrees of technical skills and diagnostic experience. A limitation of this study is our inability to control for variability among personnel making the initial diagnosis. Because of the low number of samples from certain sites in the study, we were unable to obtain reliable statistical comparison of one site's personnel's accuracy in diagnosis versus another. One suggestion for future studies would be to send known leishmaniasis positive and negative microscopic samples to each site to serve as a control for diagnostic accuracy.

Finally, we do not believe that the 82 excluded cases compromised the generalizability of our results. These patients were military individuals enrolled in a survival course at sites in El Alamo and Alto Amazonas in Peru. During the time of their infection, as part of the military training, the military personnel were purposely exposed to extremely poor sanitary conditions.⁴¹ These conditions would be abnormal for the average rural citizen exposed to leishmaniasis and, thus, the inclusion of these samples would likely jeopardize the generalizability of our findings. If these individuals had contracted the disease while on military duty, not during an artificially created environment, they certainly would have been included in the analysis.

In conclusion, there exists a need for the development and implementation of high-sensitivity, low-cost CL diagnostic tests, especially in resource-poor settings. In the meantime, certain measures, including highly qualified and trained professionals collecting samples, standardized diagnostic protocols, and uniformity of clinical criteria, should be implemented to minimize the incidence of false-negative diagnoses. Another potential outcome of this study may involve preemptive referral of patients with risk factors for false-negative diagnoses to facilities that can perform more sensitive diagnostic tests (e.g., PCR) to prevent detrimental delays in diagnosis and treatment. In addition, closer and increased patient follow-up should continue to contribute to ongoing surveillance of the accuracy of the available diagnostic tests. These processes are vital in not only preventing more patients from receiving incorrect diagnoses and not receiving the treatment they require but also in tracking the distribution of *Leishmania* parasites and vector populations, which may be rapidly evolving because of climate change and other factors.⁴²

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