Clinical and Demographic Stratification of Test Performance: A Pooled Analysis of Five Laboratory Diagnostic Methods for American Cutaneous Leishmaniasis

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Abstract. We evaluated performance characteristics of five diagnostic methods for cutaneous leishmaniasis. Patients who came to the Leishmania Clinic of Hospital Nacional Cayetano Heredia in Lima, Peru, were enrolled in the study. Lesion smears, culture, microculture, polymerase chain reaction (PCR), and leishmanin skin test (LST) were performed. A total of 145 patients with 202 lesions were enrolled: 114 patients with 161 lesions fulfilled criteria for cutaneous leishmaniasis. Sensitivity and specificity were 57.8% (95% confidence interval [CI] = 50.2–65.4%) and 100.0% for culture, 78.3% (95% CI = 71.9–84.7%) and 100.0% for microculture, 71.4% (95% CI = 64.4–78.4%) and 100.0% for smears, 78.2% (95% CI = 70.6–85.8%) and 77.4% (95% CI = 62.7–92.1%) for LST, and 96.9% (95% CI = 94.2–99.6%) and 65.9% (95% CI = 51.4–80.4%) for PCR. PCR was more sensitive than the other assays ($P < 0.001$). Sensitivities of culture, smears, and LST varied by lesion duration and appearance. PCR offers performance advantages over other assays, irrespective of patient age, sex, lesion duration, or appearance. That clinical factors influence performance of non-molecular assays offers clinicians a patient-focused approach to diagnostic test selection.

INTRODUCTION

Cutaneous leishmaniasis (CL) occurs throughout the tropics and sub-tropics affecting up to 1.5 million persons annually,[1] with more than 10,000 cases occurring each year in Peru.[2] The predominant causative species of CL in Peru is *Leishmania* (*Viannia*) *braziliensis*, although other related New World species such as *L.* (*V.*) *peruviana* and *L.* (*V.*) *guyanensis* are also well represented.[3,4] New World CL generally necessitates treatment because of the possibility of future disfiguring mucosal involvement if left untreated.[5,6] However, definitive diagnosis of CL remains challenging, particularly in resource-limited areas where the disease is highly endemic. Although molecular tests are sensitive and becoming increasingly popular (especially in research settings),[7–11] definitive diagnosis still rests on demonstration of the parasite by microscopy or culture, both of which are widely used in clinical laboratories.[1,9,12] Where molecular methods are limited by infrastructural requirements, smears and culture are limited by poor sensitivity, the requirement for technical expertise, and in the case of culture, poor turnaround time.[9,13,14] We have previously demonstrated that microculture offers an incubation time savings compared with traditional culture, and is more sensitive than smears and traditional culture.[13,14]

Although recent studies have examined the clinical and epidemiologic correlates of treatment success,[3,15,16] few studies have examined the contribution of these factors to diagnostic test performance.[14,17,18] We have reported varying performance characteristics of assays stratified by lesion appearance, although because of small numbers, sub-analysis was underpowered.[14] We herein compare the performance characteristics of five diagnostic assays for CL with clinical and demographic stratification of test performance by a pooled analysis of three diagnostic evaluations. We demonstrate that there is significant variation in test performance overall and assays such as traditional culture, smears, and leishmanin skin test (LST) have variable performance depending on factors such as patient age and sex, as well as duration and appearance of the lesion.

METHODS

Study site. Data were collected at the Leishmania Clinic of the Instituto de Medicina Tropical Alexander Von Humboldt and Hospital Nacional Cayetano Heredia, in Lima, Peru, during January–April of each year (2007–2009) after review and approval by the Institutional Review Board of the Universidad Peruana Cayetano Heredia.

Study population. Consecutive patients who came to the Leishmania Clinic for evaluation of skin lesions were enrolled as described.[13,14] We included patients who were referred to the clinic for suspected CL, had a clinical indication for skin scraping or aspirate, and were able to provide informed consent. We excluded patients with bacterial or fungal superinfection of the lesion, and those undergoing active treatment for CL.

Diagnostic investigations. Skin scraping and aspirates were obtained for Giemsa-stained lesion smears, culture, and polymerase chain reaction (PCR) as described.[13,14] Giemsa-stained smears were prepared by using material scraped from the ulcer base and border with a sterile lancet.[13,14] Smear amastigote density was quantitated as described using the method of Chulay and Bryceson.[13,14,19]

Aspirated fluid was divided evenly in a biosafety cabinet under sterile conditions and inoculated in parallel and duplicate for culture by using two methods. In the first method, 200 μL was placed in 16 × 110 mm flat-sided tissue culture tubes (Nalge Nunc International, Rochester, NY) containing 3.0 mL modified Novy-MacNeal-Nicolle (NNN) medium (blood agar base, cat. no. 245400; Difco, Detroit, MI) with 15% defibrinated rabbit blood. In the second method, 60 μL of a 1:1 mixture of aspirate and 10–20% RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with l-glutamine, 10–20% fetal

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bovine serum, 2 mM NaHCO$_3$, and 0.25 mg/mL of biopterin (pH adjusted to 7.3) (20% RPMI 1640 medium microculture) was placed in sterile, non-heparinized 1 × 75 mm capillary tubes (Chase Scientific Glass, Rockwood, TN).[13,14] After inoculation, capillary tubes were sealed with commercially available capillary tube sealant (Fisher Scientific, Ottawa, Ontario, Canada). Cultures were incubated and examined for 21 days before considered negative. For the 2009 study period, modifications of the microculture method compared with previous methods included supplementation of 20% RPMI 1640 medium with 0.25 mg/mL of biopterin (Sigma-Aldrich, St. Louis, MO).

PCR of lesion scrapings and aspirates was performed as described by using primers specific for *Leishmania* (*Viannia*) complex kinetoplast DNA (kDNA).[13,14,20,21] Briefly, kDNA isolation was performed by using the phenol/chloroform/isoamyl alcohol method, and the PCR was performed using the HotStar Taq DNA Polymerase Kit (Qiagen, Lima, Peru) in a final volume of 25 μL. The *Leishmania* (*Viannia*)–specific primer had the following sequences: MP1-L (fwd) 5′-TACTCCCCGACATGCCTCTG-3′ and MP3-H (rev) 5′-GAACGGGGTTTCTGTATGC-3′, and generated a 70-basepair product.[13,14,20,21] Amplicons were visualized by electrophoresis on 4% agarose gels (Promega, Lima, Peru) and staining with ethidium bromide. Application and interpretation of LST by using 0.1 mL of sterile, heat-killed promastigote lysate in 0.005% thimerosal was performed as described.[13,14]

Composite reference standard. In each individual study,[13,14] the composite reference standard against which each diagnostic test was compared was two of four tests with positive results, where tests refer to LST, smears, culture by either method, and PCR. For evaluation of performance characteristics of each assay, the lesion was the unit of analysis, as described.[13,14] However, because the LST was used in a per patient fashion, the patient was the unit of analysis for the LST.

Statistical analysis. Data were stored in and extracted from a structured query language database. Primary outcomes were sensitivity and specificity, and the secondary outcome was time to culture positivity. Descriptive statistics (mean, SD, median, range) were calculated for continuous variables, and differences were compared by using two-tailed *t*-tests. Categorical variables were quantitated by proportions, and differences between the groups were compared using Yate's corrected chi-square analysis. Lesions were categorized as acute (duration ≤ 3 months); subacute (duration > 3 to < 12 months); and chronic (duration ≥ 12 months). For test performance stratification analysis, patient age was categorized as ≤ 12 years, > 12 to < 18 years, 18 to < 65 years; and ≥ 65 years. Performance characteristics of each assay were compared by using the z-test. Statistical analyses were performed by using SigmaStat 2.03 software (SPSS Inc., Chicago, IL). Level of significance was set at *P* < 0.05.

RESULTS

Over the three study periods, 145 patients with 202 skin lesions were enrolled: 87 males and 58 females. Median age was 26 years (range = 7 months to 89 years), and median duration of exposure in the risk area was 36 months (range = 1 day to 88 years). Median duration of lesions was 3 months (range = 3 weeks to 27 years). Sixty-two patients (43%) had multiple lesions, and the median number of lesions per patient was

1 (range = 1–20). Five patients (3%) had evidence of simultaneous mucosal and cutaneous involvement. Most skin lesions were ulcerative (84%), and 9% and 7% had a nodular or verrucous appearance, respectively. Using the composite reference standard applied in each study (at least two of four positive test results), we determined that 161 lesions (80%) fulfilled criteria for a diagnosis of CL. A total of 185 lesions (92%) from 133 patients showed positive results by at least one test, 133 (66%) lesions from 97 patients were positive by at least three or more tests, and 56 (28%) lesions from 35 patients were positive by all five tests.

Primary outcomes. *Culture.* Of 161 lesions from 114 patients with positive results for at least two of four diagnostic tests, 127 were culture positive: 126 by microculture and 93 by traditional culture. The overall sensitivity and specificity of microculture was 78.3% (95% confidence interval [CI] = 71.9–84.7%), and 100%, respectively, and the sensitivity and specificity of traditional culture was 57.8% (95% CI = 50.2–65.4%) and 100%, respectively (*P* < 0.001) (Table 1). When the individual patient was used as the unit of analysis, sensitivities did not change appreciably from the per lesion analysis, and remained statistically significant (microculture versus traditional culture; *P* < 0.001).

Smears. A total of 115 lesions from 83 patients were positive by smears, giving a sensitivity of 71.4% (95% CI = 64.4–78.4%) and specificity of 100% (Table 1). In lesions that were smear positive, median amastigote density was grade 2 (1–10 amastigotes/100 high-power microscopic fields [hpfs)]). In lesions that fulfilled reference standard criteria, median smear density was higher in those that were also culture positive (density grade = 3, 1–10 amastigotes/10 hpfs) compared with those that were culture negative (density grade = 1, 1–10 amastigotes/1,000 hpfs) (*P* < 0.001). Results did not differ in the per patient analysis.

Polymerase chain reaction. A total of 170 lesions from 121 patients were positive by PCR, giving a sensitivity of 96.9% (95% CI = 94.2–99.6%) (Table 1). However, only 156 lesions fulfilled reference standard criteria. Thus, for 14 lesions (12 patients), PCR had the only positive test result. Compared with the reference standard, the specificity of PCR was 66% (95% CI = 51.4–80.4%) (Table 1). Overall, kDNA PCR had superior sensitivity to all other assays (*P* < 0.001). Sensitivity

TABLE 1
Analysis of five diagnostic tests used for evaluation of 145 patients with 202 lesions suspected to be those of cutaneous leishmaniasis, Lima, Peru*

Assay	No. positive	No. negative	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
LST†‡	93	48	78.2§	77.4	92.5	50.0
Smears	115	87	71.4¶	100.0	100.0	47.1
Microculture in RPMI 1640 medium	126	76	78.3#	100.0	100.0	53.9
Traditional culture in NNN medium	93	109	57.8	100.0	100.0	37.6
kDNA PCR of scrapings and aspirates	170	32	96.9**	65.9	91.8	84.4

*PPV = positive predictive value; NPV = negative predictive value; LST = leishmanin skin test; NNN = Novy-MacNeal-Nicolle; kDNA = kinetoplast DNA; PCR = polymerase chain reaction.
†Four persons did not undergo leishmanin skin testing.
‡Per patient analysis.
§*P* < 0.001 vs. traditional culture in NNN medium.
¶*P* = 0.008 vs. traditional culture in NNN medium.
#*P* < 0.001 vs. traditional culture in NNN medium.
**P* < 0.001 vs. traditional culture in NNN medium, microculture in RPMI 1640 medium, LST, and smears.

and specificity of the kDNA PCR remained the same in the per patient analysis.

Leishmanin skin test. Patients were the unit of analysis and 93 had a positive LST result, giving a sensitivity of 78.2% (95% CI = 70.6–85.8%) and specificity of 77.4% (95% CI = 62.7–92.1%) (Table 1).

Stratification by sex. The sensitivity of the LST was greater in males than in females: 87.7% (95% CI = 79.9–95.5%) versus 64.4% (95% CI = 50.6–78.2%) (P = 0.002). However, the specificity of the LST was higher in females: 100% versus 63.2% (95% CI = 41.5–84.9%) (P < 0.001). The positive predictive value (PPV) of the LST was also higher in females because of fewer false-positive results: 100% versus 89.1% (P = 0.024). Conversely, smear sensitivity was higher in females than in males: 81.3% (95% CI = 71.4–91.2%) versus 65.7% (95% CI = 56.5–74.9%) (P = 0.027). Smears had a specificity and PPV of 100% in males and females. No differences were observed between males and females for lesion duration (P = 0.537) or appearance (P = 0.15). Performance of traditional culture, microculture, and PCR did not vary by sex.

Stratification by age. The sensitivity of the LST was higher for persons > 12–18 years of age (sensitivity = 100%) than for persons ≤ 12 years of age (sensitivity = 63.2%, 95% CI = 42.6–83.8%) (P = 0.011) or for persons ≥ 65 years of age (sensitivity = 60%, 95% CI = 29.6–90.4%) (P = 0.016). Lesion duration was similar across all age groups (P = 0.697 by one-way analysis of variance). For persons > 12–18 years of age, the LST and PCR were equally sensitive (100%) and showed higher performance than traditional culture (52.0%, 95% CI = 32.4–71.6%) (P = 0.002 and P < 0.001, respectively) and smears (72.0%, 95% CI = 54.4–89.6%) (P = 0.039 and P = 0.008, respectively) and a trend towards superior performance compared with microculture (76.0%, 95% CI = 59.3–92.7%) (P = 0.069 for LST) and superior performance for PCR (P = 0.019). Smear performance was higher in persons ≥ 65 years of age than in persons ≤ 12 years of age: sensitivity 93.3% (95% CI = 80.6–100.0%) versus 60.7% (95% CI = 42.6–78.8%) (P = 0.034). Performance of traditional culture, microculture, and PCR did not vary by age.

Stratification by lesion duration. Sensitivity of traditional culture was highest for acute lesions (68.3%, 95% CI = 59.4–77.2%) than for subacute lesions (50%, 95% CI = 32.7–67.3%) (P = 0.052) or chronic lesions (24%, 95% CI = 7.3–40.7%) (P < 0.001) (Table 2). Sensitivity of microculture was not affected by lesion duration (Table 2). As with traditional culture, smears were more sensitive for acute lesions than for chronic lesions (78.8%, 95% CI = 70.9–86.7% versus 44%, 95% CI = 24.5–63.5%) (P < 0.001) and showed a trend

toward significance for subacute lesions versus chronic lesions (68.8%, 95% CI = 52.7–84.9% versus 44%, 95% CI = 24.5–63.5%) (P = 0.054) (Table 2). The sensitivity of PCR did not differ for different categories of lesion duration. The LST was particularly sensitive in for chronic lesions and showed superior performance compared with smears and traditional culture (P < 0.001). The LST was as sensitive as microculture for all lesion durations, and equally sensitive as PCR for chronic lesions (Table 2).

Stratification by lesion appearance. For ulcerative lesions, the sensitivity of PCR was higher than that of every other assay (P < 0.001) (Table 3). Microculture (sensitivity = 79.5%, 95% CI = 72.6–86.4%) and LST (sensitivity = 79.8%, 95% CI = 71.4–88.2%) were more sensitive than traditional culture (sensitivity = 62.9%, 95% CI = 54.7–71.1%) for ulcerative lesions (P = 0.001 and P = 0.003, respectively) (Table 3). For non-ulcerative lesions (nodular or verrucous), the sensitivity of PCR (93.1%, 95% CI = 83.9–100%) was higher than that of smears (sensitivity = 65.5%, 95% CI = 48.2–82.8%) (P = 0.016), traditional culture (sensitivity = 34.5%, 95% CI = 17.2–51.8%) (P < 0.001), and showed a trend trended towards superiority over microculture (sensitivity = 72.4%, 95% CI = 56.1–88.7%) (P = 0.063) and the LST (sensitivity = 71.4%, 95% CI = 52.5–90.3%) (P = 0.073). As for ulcers, microculture had a greater sensitivity than traditional culture for non-ulcerative lesions (P = 0.005) (Table 3). Smears, traditional culture, and microculture showed poor performance in the subset of non-ulcerative lesions that were verrucous and had the following sensitivities: smears = 58.3% (95% CI = 30.4–86.2%), traditional culture = 16.7% (95% CI = 0–37.8%), and microculture = 58.3% (95% CI = 30.4–86.2%). The sensitivity of traditional culture for ulcerative lesions was significantly higher than that of the same assay for non-ulcerative lesions (P = 0.005) (Table 3).

Secondary Outcomes. *Time to culture positivity.* Median time to culture positivity was 3 days by microculture (range = 1–10 days), and 5 days by traditional culture (range = 2–14 days) (P < 0.001). When the individual patient was used as the unit of analysis, median time to culture positivity was the same and remained statistically significant (P < 0.001).

DISCUSSION

We have established in a pooled analysis of three diagnostic evaluations that PCR and microculture show the greatest sensitivity among commonly used assays for the diagnosis of CL. Although the specificity of PCR appeared sub-optimal compared with that of the other tests, this observation likely

TABLE 2

Sensitivity and specificity of five diagnostic tests used for evaluation of 145 patients with 202 lesions suspected to those of cutaneous leishmaniasis and stratified by lesion duration, Lima, Peru*

Assay	Acute lesions (duration ≤ 3 months) (n = 124 lesions from 88 patients)		Sub-acute lesions (duration > 3 to < 12 months) (n = 42 lesions from 30 patients)		Chronic lesions (duration ≥ 12 months) (n = 33 lesions from 24 patients)	
	Sensitivity, %	Specificity, %	Sensitivity, %	Specificity, %	Sensitivity, %	Specificity, %
LST†‡	75.0	78.6	78.3	83.3	93.3	75.0
Smears	78.8	100.0	68.8	100.0	44.0	100.0
Microculture in RPMI 1640 medium	79.8	100.0	71.9	100.0	80.0	100.0
Traditional Culture in NNN medium	68.3	100.0	50.0	100.0	24.0	100.0
kDNA PCR of scrapings and Aspirates	99.0	65.0	96.9	70.0	88.0	75.0

* LST = leishmanin skin test; NNN = Novy-MacNeal-Nicolle; kDNA = kinetoplast DNA; PCR = polymerase chain reaction.
† Four persons did not undergo leishmanin skin testing.
‡ Per patient analysis.

Sensitivity and specificity of five diagnostic tests used for evaluation of 145 patients with 202 lesions suspected to be those of cutaneous leishmaniasis and stratified by lesion appearance, Lima, Peru*

Assay	Ulcerative lesions (n = 170 lesions from 122 patients)		Non-ulcerative lesions (nodular or verrucous appearance) (n = 32 lesions from 23 patients)	
	Sensitivity, %	Specificity, %	Sensitivity, %	Specificity, %
LST†‡	79.8	80.0	71.4	50.0
Smears	72.7	100.0	65.5	100.0
Microculture in RPMI 1640 medium	79.5	100.0	72.4	100.0
Traditional culture in NNN medium	62.9	100.0	34.5	100.0
kDNA PCR of scrapings and aspirates	97.7	63.2	93.1	100.0

*LST = leishmanin skin test; NNN = Novy-MacNeal-Nicolle; kDNA = kinetoplast DNA; PCR = polymerase chain reaction.
†Four persons did not undergo leishmanin skin testing.
‡Per patient analysis.

reflects the definition of our consensus reference standard (i.e., two of four tests with positive results), rather than a poorer specificity. The 14 lesions for 12 patients that were positive only by PCR and therefore deemed as false-positive results by our reference standard, were all clinically suspected to indicate leishmaniasis, and may have been caused by *Leishmania*. A possible explanation for this low specificity is performance of PCR beyond the threshold of parasite detection for the other assays, which leads to a higher number of false-positive results. This is certainly a well-described phenomenon and an acknowledged limitation of comparative diagnostic evaluations in the setting of a sub-optimally performing reference standard.[22] Although the specificity of the PCR may have been falsely decreased in this analysis, the sensitivity and rapid turn-around time make it an extremely attractive alternative to other diagnostic methods, such as smears and LST, which do not rely on parasite isolation.

Microculture has a clear performance advantage over traditional culture. Not only is microculture more sensitive, but it offers the laboratory a two-day incubation saving over traditional culture, which is important for diseases such as CL, in which empiric therapy is rarely administered before definitive diagnosis due to drug toxicity.[6,9] Because of the small size of capillary tubes and the minuscule volume of required media, microculture also offers an economic advantage over traditional culture.[13,14,23] One major limitation of microculture is the inability to perform further downstream testing once the organism is isolated. We have observed that promastigotes will replicate within capillary tubes to high densities and remain viable for up to six weeks (Boggild AK, unpublished data), however, there is no safe way to extract the cultured organisms, and we would not advocate attempting such a maneuver because of the inherent risks of handling blood and body fluid and the potential transmissibility of the infectious promastigote stage should an accident occur. In light of this limitation, microculture may be viewed as a superior extension of smears in that it is highly specific and rapid, but fails to provide a source of the organism.

It has long been recognized that the performance of diagnostic tests for leishmaniasis varies according to the clinical spectrum of disease (from visceral to mucocutaneous), with parasitologic methods such as smears and culture favored for detection of polyparasitic forms of the disease, and non-parasitological methods such as histopathologic analysis and molecular detection favored for detection of oligoparasitic forms of the disease.[9] However, little attention has been paid to the contribution of patient demographic and other clinical factors on test performance characteristics. We have demonstrated a significant performance bias of several assays based on patient age, sex, lesion duration, and lesion appearance.

Sex-based stratification of assay performance showed superior sensitivity of the LST in males than in with females when we used the consensus reference standard of two of four positive test results. However, specificity and PPV of the LST were superior in females because of fewer false-positive results. Conversely, lesion smears showed the opposite performance pattern: sensitivity was higher in females than in males, but equally specific (100%) in both sexes. These findings were independent of lesion duration or lesion appearance, although may have been influenced by other confounders not analyzed herein. Interestingly, our findings for the LST are in contrast with those of Sadeghian and others, who found no relationship between age, sex, and lesion duration with LST positivity,[17] which suggested that other confounding factors may have been contributory in our study.

PCR demonstrated excellent sensitivity for ulcers, and was superior to all other assays in the overall analysis. Although the sensitivity and specificity of PCR were also excellent for non-ulcerative lesions, PCR did not afford a statistically significant performance advantage over LST in patients with lesions ≥ 12 months of age. Traditional culture and smears had fairly poor performance for chronic and non-ulcerative lesions. Conversely, the LST was a particularly sensitive assay for patients with chronic lesions, and equally sensitive as the PCR in this group of patients. Positivity of the LST is correlated with a T_H1-type cytokine profile (with a predominance of upregulation of interferon-γ) *in vitro*.[24–26] Within the first 60 days of CL infection, interferon-γ is downregulated by increased levels of interleukin-10, which may enable high parasite replication in the lesion.[24,25,27] This finding may explain the observed pattern of high LST positivity in patients with older lesions and higher smear positivity in patients with younger lesions.

Our data provide supportive evidence for an algorithmic approach to CL diagnosis based on demographic and clinical factors such as lesion age and appearance (Table 4). Patients with lesions ≥ 12 months of age and/or nodular or verrucous in appearance are unlikely to benefit from smears and traditional culture, which are two of the most commonly used diagnostic tests for this disease. Rather, this particular clinical pattern is more likely to yield positive results though a combination of testing by LST and PCR. Conversely, the LST alone would not be favored in persons in the youngest and oldest age groups or in females. Of the clinical and demographic factors analyzed, smears had the best performance in females, those ≥ 65 years of age, and those with ulcers. Thus, smears could be a more useful diagnostic tool in patients fulfilling these criteria.

Cutaneous leishmaniasis remains a challenge to diagnose and each of the commonly used diagnostic assays has advantages and limitations. Through this analysis, we have provided clinicians and laboratorians with a robust assessment of assay performance characteristics in a large group of demographically and clinically heterogeneous patients from Peru who came to a specialized leishmaniasis clinic. Our findings may

Table 4

Clinical utility of five laboratory tests used in the diagnosis of cutaneous leishmaniasis on the basis of stratification of patients by age, sex, lesion duration, and lesion appearance, Lima, Peru

Test	Clinical utility
Leishmanin skin test	Best sensitivity for males, those with chronic lesions (duration ≥ 12 months), and for persons 12–18 years of age; superior to traditional culture for non-ulcerative lesions; equally sensitive as the polymerase chain reaction for persons 12–18 years of age and for chronic lesions (duration ≥ 12 months); poor sensitivity for females, children ≤ 12 years of age, and adults ≥ 65 years of age
Giemsa-stained lesion smears	Highest sensitivity for females, persons ≥ 65 years of age, and persons with ulcers; poor sensitivity for chronic (duration ≥ 12 months) or non-ulcerative lesions; 100% specificity across all groups
Microculture in RPMI 1640 medium	Stable performance, including 100% specificity, across age groups, sex, lesion duration, and lesion appearance
Traditional culture in Novy-MacNeal-Nicolle	Only minimally useful for ulcers and lesions with a duration ≤ 3 months; extremely poor sensitivity for non-ulcerative lesions and for lesions with a duration ≥ 12 months although 100% specific
Kinetoplast DNA polymerase chain reaction	Highest sensitivity of all assays with little variability; performs well across age groups, sex, lesion duration, and lesion appearance

likely be applied to other species of *Leishmania* in other geographic settings, given that PCR and microculture have been shown to be superior to traditional diagnostic methods in Old World CL.[23] We have attempted to provide clinicians with an evidence-based approach to clinical specimen sampling that takes into account factors such as patient age, sex, lesion duration, and lesion appearance, which we hope will improve the diagnostic yield of testing and reduce the use of tests with inferior performance in certain patient populations.

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