Leishmania OligoC-TesT as a Simple, Rapid, and Standardized Tool for Molecular Diagnosis of Cutaneous Leishmaniasis in Peru

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Molecular methods such as PCR have become attractive tools for diagnosis of cutaneous leishmaniasis (CL), both for their high sensitivity and for their specificity. However, their practical use in routine diagnosis is limited due to the infrastructural requirements and the lack of any standardization. Recently, a simplified and standardized PCR format for molecular detection of Leishmania was developed. The Leishmania OligoC-TesT is based on simple and rapid detection using a dipstick with PCR-amplified Leishmania DNA. In this study, we estimated the diagnostic accuracy of the Leishmania OligoC-TesT for 61 specimens from 44 CL-suspected patients presenting at the leishmaniasis clinic of the Instituto de Medicina Tropical Alexander von Humboldt, Peru. On the basis of parasitological detection and the leishmanin skin test (LST), patients were classified as (i) confirmed CL cases, (ii) LST-positive cases, and (iii) LST-negative cases. The sensitivities of the Leishmania OligoC-TesT was 74% (95% confidence interval [CI], 60.5% to 84.1%) for lesion aspirates and 92% (95% CI, 81.2% to 96.9%) for scrapings. A significantly higher sensitivity was observed with a conventional PCR targeting the kinetoplast DNA on the aspirates (94%) (P = 0.001), while there was no significant difference in sensitivity for the lesion scrapings (88%) (P = 0.317). In addition, the Leishmania OligoC-TesT was evaluated for 13 CL-suspected patients in two different peripheral health centers in the central jungle of Peru. Our findings clearly indicate the high accuracy of the Leishmania OligoC-TesT for lesion scrapings for simple and rapid molecular diagnosis of CL in Peru.

Leishmaniasis is a vector-borne disease caused by obligatory intracellular parasites of the genus Leishmania. Several clinical manifestations are classified under the term leishmaniasis, but three are the most prominent: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL), which result from replication of parasites in macrophages in the internal organs, dermis, and naso-oropharyngeal mucosa, respectively (15).

In Latin America, CL and MCL are important health problems, and Brazil and Peru are the two most affected countries (9). An increase of cases has been reported for Colombia, Ecuador, and Argentina (4, 25, 26). Human-made risk factors, such as migration, urbanization, and deforestation, likely contribute to the spread of the disease (10).

Over 10,000 CL cases per year are reported to occur in Peru, and more than a million people are at risk for infection (11, 22). Furthermore, this disease is endemic in 70% of Peruvian territory, causing high morbidity, lifelong scars, and major health problems for many communities (19).

Diagnosing CL is challenging because of its wide spectrum of clinical presentations. Lesions may vary in severity, clinical appearance, and duration (23). Moreover, differential diagnosis with other cutaneous diseases is often difficult (14, 15). In addition, CL can be caused by different Leishmania species. In Peru, the disease is mainly caused by Leishmania (Vianna) braziliensis, Leishmania (Vianna) peruviana, and Leishmania (Vianna) guyanensis, but Leishmania (Vianna) lainsoni and Leishmania (Leishmania) amazonensis infections have also been reported (1, 18).

Routine diagnosis of CL is still based on demonstration of amastigotes in skin lesion scrapings through microscopic analysis of direct smears or prior in vitro culture of the parasite (15, 23). Both methods require skilled personnel, and their sensitivities tend to be low and variable (12, 21, 27). Furthermore, in vitro culture is cumbersome and time-consuming. The leishmanin skin test (LST) detects cell-mediated immunity and is frequently used in Peru to support clinical diagnosis of CL. However, it cannot distinguish between past and present infections (12).

The PCR is a useful tool for detection of Leishmania parasites in clinical specimens, since high sensitivity and specificity have been reported. Attractive PCR targets are high-copy-number sequences, such as kinetoplast DNA (kDNA) (2, 8, 30) and the ribosomal small subunit (20, 28). Several PCR formats have been designed, but there is actually a demand for simplified and standardized approaches (24). Access to sophisticated
equipment such as real-time PCR machines is often limited in Peru. Recently, a simple and rapid dipstick format for detection of amplified Leishmania DNA was developed (Leishmania OligoC-TesT) (7). The test is based on PCR amplification of a small sequence of the 18S rRNA gene followed by visualization of the PCR products on a dipstick by hybridization with a gold-conjugated probe. PCR product detection can be performed in 10 min, and no equipment other than a heating block and a pipette is needed. The test is a promising “low-tech” standardized PCR application for diagnosis of leishmaniasis and can be applied in a mid- to low-level-equipped laboratory (6).

In this report, we estimated the sensitivity of the Leishmania OligoC-TesT for 61 skin lesion scrapings from 44 Peruvian patients suspected of having CL. To assess the performance and to demonstrate the applicability of the test in low-level-equipped laboratories, two trials in rural hospitals in the Peruvian jungle were conducted.

**MATERIALS AND METHODS**

**Patients.** Informed consent was obtained from the participants in the study. Ethical clearance for the study was obtained from the institutional review board of the Universidad Peruana Cayetano Heredia in Lima, Peru. DNA extracts of aspirates and scrapings of 61 lesions from 44 patients were obtained from a previous study at the leishmaniasis clinic of the Instituto de Medicina Tropical Alexander von Humboldt in Lima, Peru, in the period between February and April 2007 (3). DNA extracts were stored at -70°C until testing in the current study, 6 months after initial specimen taking.

In addition, the test was evaluated for CL-suspected patients admitted at the peripheral health centers of San Martín de Pangoa and Kiteni in the Junín and Cusco regions, respectively. Both regions are among those with the highest CL endemicity in Peru, including MCL for the latter. In the Junín region, CL is mainly caused by L. (V.) guyanensis (1, 18), while L. braziliensis accounts for most CL and MCL cases in the Cusco region (18). Lesion scrapings were collected from 4 CL-suspected patients in San Martín de Pangoa in February 2008 and from 13 CL-suspected patients in Kiteni in July 2008.

**Reference tests.** (i) Smears. Tissue was scraped from the lesion border by using a sterile lancet and spread on a glass slide. The slides were air dried, fixed in methanol, and stained with Giemsa, followed by microscopic analysis under light microscopy. Amastigotes in positive smears at the leishmaniasis clinic in Lima were quantified and classified into six grades according to the method of Chulay and Bryceson (5).

(ii) Culture. In vitro culture of parasites was performed as described by Boggild et al. (3). Three different culture methods were used: (i) conventional culture in 10% RPMI medium, (ii) microculture method with the same medium, and (iii) culture in modified NNN (Noy-MacNeal-Nicolle) medium with 15% defibrinated rabbit blood. The culture was considered positive if parasites were observed by microscopic analysis with one or more of the three culture techniques.

(iii) LST. Leishmanian antigen (0.1 ml), prepared from the L. (V.) guyanensis strain LP52 (IPRN/PE/87/Lp52) at the Instituto de Medicina Tropical Alexander von Humboldt in Lima, was injected in the volar surface of the forearm, and induration and erythema were measured after 48 h. The LST result was considered positive if the diameter of the induration was 5 mm or more (31).

**Patient classification.** A patient was classified as (i) a confirmed CL patient if positive by smear or culture, (ii) an LST-positive patient if negative by smear and culture but positive by LST, and (iii) an LST-negative patient if negative by smear and culture and LST.

**DNA extraction.** DNA of lesion scraping specimens from the recruited patients at San Martín de Pangoa and Kiteni was extracted according to the simplified procedure described by López et al. (17). Briefly, lancets with scraped tissue were immersed in 100 μl of lysis buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1.000 mM NaCl) and incubated at 94°C for 30 min. Lysed specimens were directly used in PCR.

**kDNA PCR.** A Leishmania (V-) braziliensis complex-specific kDNA PCR was performed on the lesion aspirate and scraping specimens as described by López et al. (17).

**RESULTS**

Leishmania OligoC-TesT at the leishmaniasis clinic in Lima. (i) Confirmed CL patients (n = 35). Fifty lesion scrapings from 35 patients were classified in the confirmed-CL category on the basis of the parasitological detection methods (Table 1). Positive LST results were observed for 27 of the 33 patients for which LST results were available, indicating a sensitivity of 81.8%, with a 95% confidence interval (CI) of 65.6% to 91.4%, scored by Wilson’s method (32). The DNA extracts from the lesion aspirates were positive by the Leishmania OligoC-TesT for 37 of the 50 specimens, while 47 were positive with the kDNA PCR. Hence, the sensitivities of the Leishmania OligoC-TesT and kDNA PCR for the 50 aspirate specimens are 74% (95% CI, 60.5% to 84.1%) and 94% (95% CI, 83.8% to 98%), respectively. When tested on DNA extracted from lesion scrapings, 46 of the 50 specimens were found to be positive by the Leishmania OligoC-TesT, while 44 were found to be positive with the kDNA PCR, indicating sensitivities of 92% (95% CI, 81.2% to 96.9%) and 88% (95% CI, 76.2% to 94.4%), respectively. Out of the four OligoC-TesT-negative lesion scrapings, three showed the lowest amastigote load (grade 1), while the fourth was negative by smear but positive by culture. Using the McNemar chi-square method, we observed that the kDNA PCR is significantly more sensitive for aspirate specimens (P = 0.001) but that the sensitivities of the two methods did not differ for scrapings (P = 0.317).

(ii) LST-positive patients (n = 2). The lesion aspirates from the two LST-positive patients were positive by kDNA PCR, but those from both were negative by the Leishmania OligoC-TesT. Upon testing with lesion scrapings, a positive kDNA PCR result was observed in one of the two cases, and positive Leishmania OligoC-TesT results were observed in both cases.

(iii) LST-negative patients (n = 7). Nine lesion scrapings from seven LST-negative patients were negative by smear and culture. Seven aspirate specimens were positive by kDNA PCR, and four were positive by the Leishmania OligoC-TesT.
confirmed CL patients and two of the five nonconfirmed suspected patients at San Martín de Pangoa were positive by the Leishmania OligoC-TesT results. No kDNA PCR was performed at Kiteni.

**DISCUSSION**

Molecular approaches have shown great potential for diagnosis of leishmaniasis, follow-up after treatment, disease control, and epidemiology (1, 8, 19, 24, 29). Recently, the *Leishmania* OligoC-TesT was introduced as an innovative PCR format for simplified and standardized molecular diagnosis of *Leishmania* infections (7). We have demonstrated that the *Leishmania* OligoC-TesT offers performance comparable to that of conventional kDNA PCR for lesion scrapings in cases of suspected and confirmed CL in Peru.

On lesion aspirate specimens, we observed lower sensitivity for the *Leishmania* OligoC-TesT than for kDNA PCR (74% versus 92%). This could be explained by the lower number of copies of the 18S rRNA gene (10 to 100 copies) than of the minicircles (10,000 copies) in the kDNA. However, when lesion scrapings from the same lesions were tested, the sensitivity of the *Leishmania* OligoC-TesT was found to be 92% and the sensitivity of the kDNA PCR was found to be 88%. The same trend was observed with the two LST-positive patients, where lesion scrapings from both were negative by the *Leishmania* OligoC-TesT with aspirates but positive with lesion scrapings. In contrast to lesion aspirates, scrapings may contain fewer PCR-inhibiting factors but also fewer parasites. The *Leishmania* OligoC-TesT is probably more liable to inhibition than the kDNA PCR, giving rise to a higher sensitivity for lesion scrapings in spite of the lower parasite load. Moreover, our results confirm the findings of Garcia et al. (13), who reported on the use of lesion scrapings for parasite species identification by conventional PCR. Hence, this favors the use of lesion scrapings over aspirates and biopsy specimens, since scrapings are much less invasive. The positive PCR results in the LST-negative specimen group are probably from patients with low-level cell-mediated immunity responses to the infection or from recent skin lesions. The LST is a more sensitive tool in the setting of chronic infection (31). Contamination during PCR is unlikely since negative controls remained negative and since positive results were observed with two PCRs targeting two different DNA sequences. The high sensitivity of the LST for the confirmed CL group (81.8%) supports the valuable contribution of LST to CL diagnosis.

Four scrapings were positive by kDNA PCR and three by the *Leishmania* OligoC-TesT.

**Leishmania OligoC-TesT at the peripheral health centers.**

The *Leishmania* OligoC-TesT was evaluated for 17 CL-suspected patients at two peripheral health centers in areas where CL is highly endemic (Fig. 1). Lesion scrapings from 2 of the 4 suspected patients at San Martín de Pangoa and from 8 of the 13 suspected patients at Kiteni showed parasites during microscopic analysis of lesion tissue smears and/or in vitro culture of parasites in modified NNN medium with 15% defibrinated rabbit blood. Scrapings from both confirmed CL patients at San Martín de Pangoa were positive by the *Leishmania* OligoC-TesT, while those from the two nonconfirmed patients were negative by the *Leishmania* OligoC-TesT. The latter were additionally tested with kDNA PCR, and negative test results were observed as well. Lesion scrapings from seven of the eight confirmed CL patients and two of the five nonconfirmed suspected patients at Kiteni showed positive *Leishmania* OligoC-TesT results. No kDNA PCR was performed at Kiteni.

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