

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

Diego Espinosa,^{1*} Andrea K. Boggild,² Stijn Deborggraeve,³ Thierry Laurent,⁴ Cristian Valencia,¹ Rosa Pacheco,⁵ César Miranda-Verástegui,¹ Alejandro Llanos-Cuentas,¹ Thierry Leclipteux,⁴ Jean-Claude Dujardin,³ Philippe Büscher,³ and Jorge Arévalo^{1,6}

Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru¹; Tropical Diseases Unit, Division of Infectious Diseases, University Health Network, Toronto General Hospital, Toronto, Canada²; Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium³; Coris BioConcept, Gembloux, Belgium⁴; Universidad Nacional San Antonio Abad del Cusco, Cusco, Peru⁵; and Departamento de Bioquímica, Biología Molecular y Farmacología, Facultad de Ciencias, Universidad Peruana Cayetano Heredia, Lima, Peru⁶

Received 6 February 2009/Returned for modification 20 May 2009/Accepted 16 June 2009

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-oro-pharyngeal 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* (*Viannia*) *braziliensis*, *Leishmania* (*Viannia*) *peruviana*, and *Leishmania* (*Viannia*) *guyanensis*, but *Leishmania* (*Viannia*) *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

* Corresponding author. Mailing address: Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Avenida Honorio Delgado 430, Lima 31, Peru. Phone: (51-1) 482-3910. Fax: (51-1) 482-3404. E-mail: diegoespinosa@gmail.com.

[∇] Published ahead of print on 24 June 2009.

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 Bryceon (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) the microculture method with the same medium, and (iii) culture in modified NNN (Novy-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. Leishmanin 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).

TABLE 1. Diagnostic accuracy of the *Leishmania* OligoC-TesT and kDNA PCR for 61 lesion specimens from 45 CL-suspected patients at the leishmaniasis clinic in Lima

Patient group	No. of lesion samples	No. (%) positive ^a			
		Lesion aspirates		Lesion scrapings	
		OligoC-TesT	kDNA PCR	OligoC-TesT	kDNA PCR
Confirmed CL	50	37	47	46	44
LST positive	2	0	2	2	1
LST negative	9	4	7	3	4

^a The sensitivities (95% CIs) of the tests were as follows: for lesion aspirates, 74% (60.5% to 84.1%) for the OligoC-TesT and 94% (83.8% to 98%) for kDNA PCR; and for lesion scrapings, 92% (81.2% to 96.9%) for the OligoC-TesT and 88% (76.2% to 84.4%) for kDNA PCR. CIs were scored by Wilson's method.

***Leishmania* OligoC-TesT.** The *Leishmania* OligoC-TesT was performed as described by Deborggraeve et al. (7). Briefly, *Leishmania* DNA was amplified by PCR and mixed with an equal volume of migration buffer preheated at 55°C . The *Leishmania* OligoC dipstick was dipped in the solution, and test results were read after 10 min of incubation at 55°C . Two hundred nanograms of *L. (V.) guyanensis* (strain IPRN/PE/87/Lp52) DNA was used as a positive control for PCR.

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.

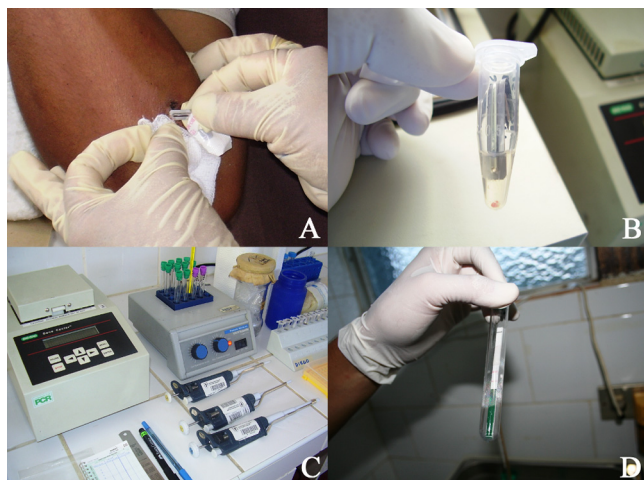


FIG. 1. (A) Lesion scraping procedure using a sterile lancet; (B) lancet with scraped lesion material in buffer prior to tissue lysis; (C) equipment used in the assay; (D) macroscopic visualization of results.

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.

DISCUSSION

Molecular approaches have shown great potential for diagnosis of leishmaniasis, follow-up after treatment, disease control, and epidemiology (1, 8, 18, 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 García 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.

The results obtained with the *Leishmania* OligoC-TesT at the peripheral health centers in the Peruvian jungle show that the *Leishmania* OligoC-TesT can be conducted in low-level-equipped laboratories. Both health centers are located in high-CL-endemicity regions characterized by impoverished populations and limited access to appropriate health care. The results obtained by the test for the two CL-suspected patients at the health center in San Martín de Pangoa were perfectly concordant with the results obtained by conventional diagnosis and were confirmed by kDNA PCR. At the health center in Kiteni, the *Leishmania* OligoC-TesT results were in agreement with conventional diagnosis for 10 of the 13 CL-suspected patients. One patient showed parasites during parasitological detection but a negative result with the *Leishmania* OligoC-TesT.

This study demonstrates the high accuracy of the *Leishmania* OligoC-TesT for noninvasive lesion scrapings. A major advantage of the test compared to parasite culture and LST is the time reduction. OligoC-TesT results are available in 5 h after the initial specimen is obtained, while culture may require up to 2 to 4 weeks and LST 2 days. Diagnosis within 1 day is important since patients often live far from the health centers and extra costs due to transport or lodging should be avoided. Furthermore, the OligoC-TesT can be produced in a kit format including a quality-controlled PCR mixture and dipsticks. This will likely enhance the integration of the PCR technique in leishmaniasis reference centers as well as in peripheral health centers in areas of endemicity. The development of a PCR dipstick which is able to discriminate among different New World *Leishmania* species would be most welcome (16), as disease progression and treatment response are species specific (1, 19).

ACKNOWLEDGMENTS

This study received financial support from the Directorate-General for Development Cooperation of the Belgian Government (framework agreement 03, project 95502) and from the European Community

through the project "Control strategies for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL) in South America: applications of molecular epidemiology" (contract INCO-CT2005-015407).

REFERENCES

- Arévalo, J., L. Ramirez, V. Adauí, M. Zimic, G. Tulliano, C. Miranda-Verástegui, M. Lazo, R. Loayza-Muro, S. De Doncker, A. Maurer, F. Chappuis, J. C. Dujardin, and A. Llanos-Cuentas. 2007. Influence of *Leishmania (Viannia)* species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. *J. Infect. Dis.* **195**:1846–1851.
- Bensoussan, E., A. Nasereddin, F. Jonas, L. F. Schnur, and C. L. Jaffe. 2006. Comparison of PCR assays for diagnostics of cutaneous leishmaniasis. *J. Clin. Microbiol.* **44**:1435–1439.
- Boggild, A. K., C. Miranda-Verástegui, D. Espinosa, J. Arévalo, V. Adauí, G. Tulliano, A. Llanos-Cuentas, and D. E. Low. 2007. Evaluation of a microculture method for the isolation of *Leishmania* parasites from cutaneous lesions in Peru. *J. Clin. Microbiol.* **45**:3680–3684.
- Calvopina, M., R. X. Armijos, and Y. Hashiguchi. 2004. Epidemiology of leishmaniasis in Ecuador: current status of knowledge. *Mem. Inst. Oswaldo Cruz* **99**:663–672.
- Chulay, J. D., and A. D. Bryceson. 1983. Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniasis. *Am. J. Trop. Med. Hyg.* **32**:475–479.
- Deborggraeve, S., F. Claes, T. Laurent, P. Mertens, T. Leclipteux, J. C. Dujardin, P. Herdewijn, and P. Büscher. 2006. Molecular dipstick test for diagnosis of sleeping sickness. *J. Clin. Microbiol.* **44**:2884–2889.
- Deborggraeve, S., T. Laurent, D. Espinosa, G. Van der Auwera, M. Mbuchi, M. Wasunna, S. El-Safi, A. A. Al-Basheer, J. Arévalo, C. Miranda-Verástegui, T. Leclipteux, P. Mertens, J. C. Dujardin, P. Herdewijn, and P. Büscher. 2008. A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. *J. Infect. Dis.* **198**:1565–1572.
- de Oliveira, C. I., A. Báfica, F. Oliveira, C. B. F. Favali, T. Correa, L. A. R. Freitas, E. Nascimento, J. M. Costa, and A. Barral. 2003. Clinical utility of polymerase chain reaction-based detection of *Leishmania* in the diagnosis of American cutaneous leishmaniasis. *Clin. Infect. Dis.* **37**:e149–e153.
- Desjeux, P. 2004. Disease watch focus: leishmaniasis. *Nat. Rev. Microbiol.* **2**:692–693.
- Desjeux, P. 2001. The increase of risk factors for leishmaniasis worldwide. *Trans. R. Soc. Trop. Med. Hyg.* **95**:239–243.
- Dirección General de Epidemiología. 2002. Anuario del sistema de vigilancia epidemiológica. Ministerio de Salud, Lima, Peru.
- Faber, W. R., L. Oskam, T. Van Gool, N. C. M. Kroon, K. J. Knegt-Junk, H. Hofwegen, A. C. van der Wal, and P. A. Kager. 2003. Value of diagnostic techniques for cutaneous leishmaniasis. *J. Am. Acad. Dermatol.* **49**:70–74.
- García, A. L., R. Parrado, S. De Doncker, H. Bermudez, and J. C. Dujardin. 2007. American tegumentary leishmaniasis: direct species identification of *Leishmania* in non-invasive clinical samples. *Trans. R. Soc. Trop. Med. Hyg.* **101**:368–371.
- Hepburn, N. C. 2003. Cutaneous leishmaniasis: an overview. *J. Postgrad. Med.* **49**:50–54.
- Herwaldt, B. L. 1999. Leishmaniasis. *Lancet* **354**:1191–1199.
- Laurent, T., G. Van der Auwera, M. Hide, P. Mertens, W. Quispe-Tintaya, S. Deborggraeve, S. De Doncker, T. Leclipteux, A. L. Bañuls, P. Büscher, and J. C. Dujardin. 2009. Identification of Old World *Leishmania* spp. by specific polymerase chain reaction amplification of cysteine proteinase B genes and rapid dipstick detection. *Diagn. Microbiol. Infect. Dis.* **63**:173–181.
- López, M., C. Orrego, M. Cangalaya, R. Inga, and J. Arévalo. 1993. Diagnosis of *Leishmania* via the polymerase chain reaction a simplified procedure for field work. *Am. J. Trop. Med. Hyg.* **49**:348–356.
- Lucas, C. M., E. D. Franke, M. I. Cachay, A. Tejada, M. E. Cruz, R. D. Kreuzer, D. C. Barker, S. H. McCann, and D. M. Watts. 1998. Geographic distribution and clinical description of leishmaniasis cases in Peru. *Am. J. Trop. Med. Hyg.* **59**:312–317.
- Llanos-Cuentas, A., G. Tulliano, R. Araujo-Castillo, C. Miranda-Verástegui, G. Santamaria-Castrellon, L. Ramirez, M. Lazo, S. De Doncker, M. Boelaert, J. Robays, J. C. Dujardin, J. Arévalo, and F. Chappuis. 2008. Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. *Clin. Infect. Dis.* **46**:223–231.
- Marfurt, J., A. Nasereddin, I. Niederwieser, C. L. Jaffe, H. P. Beck, and I. Felger. 2003. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the minixon sequence and subsequent restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **41**:3147–3153.
- Marques, M. J., A. C. Volpini, G. L. L. Machado-Coelho, J. Machado-Pinto, C. A. da Costa, W. Mayrink, O. Genaro, and A. J. Romanha. 2006. Comparison of polymerase chain reaction with other laboratory methods for diagnosis of American cutaneous leishmaniasis: diagnosis of cutaneous leishmaniasis by polymerase chain reaction. *Diagn. Microbiol. Infect. Dis.* **54**:37–43.
- Ministerio de Salud del Perú. 2000. Leishmaniasis. Módulos técnicos serie documentos monográficos. Ministerio de Salud, Lima, Peru.
- Murray, H. W., J. D. Berman, C. R. Davies, and N. Saravia. 2005. Advances in leishmaniasis. *Lancet* **366**:1561–1577.
- Reithinger, R., and J. C. Dujardin. 2007. Molecular diagnosis of leishmaniasis: current status and future applications. *J. Clin. Microbiol.* **45**:21–25.
- Rodriguez-Barraquer, I., R. Góngora, M. Prager, R. Pacheco, L. M. Montero, A. Navas, C. Ferro, M. C. Miranda, and N. G. Saravia. 2008. Etiologic agent of an epidemic of cutaneous leishmaniasis in Tolima, Colombia. *Am. J. Trop. Med. Hyg.* **78**:276–282.
- Segura, E. L., N. Juan, A. L. M. Piquin, C. A. Cuba Cuba, L. Abramo Orrego, D. McMahon-Pratt, E. E. Montamat, H. Momen, and G. Grimaldi, Jr. 2000. Molecular and biologic characterization of *Leishmania* parasites implicated in an epidemic outbreak in northwestern Argentina. *Parasitol. Res.* **86**:504–508.
- Singh, S. J. 2003. Recent advances in the diagnosis of leishmaniasis. *J. Postgrad. Med.* **49**:55–60.
- van der Meide, W. F., G. J. Schoone, W. R. Faber, J. E. Zeegelaar, H. J. C. de Vries, Y. Özbek, R. F. M. Lai A Fat, L. I. A. R. C. Coelho, M. Kassi, and H. D. F. H. Schallig. 2005. Quantitative nucleic acid sequence-based assay as a new molecular tool for detection and quantification of *Leishmania* parasites in skin biopsy samples. *J. Clin. Microbiol.* **43**:5560–5566.
- van der Meide, W. F., I. Peekel, P. P. A. M. van Thiel, H. D. F. H. Schallig, H. J. C. de Vries, J. E. Zeegelaar, and W. R. Faber. 2008. Treatment assessment by monitoring parasite load in skin biopsies from patients with cutaneous leishmaniasis, using quantitative nucleic acid sequence-based amplification. *Clin. Exp. Dermatol.* **33**:394–399.
- Weigle, K. A., L. A. Labrada, C. Lozano, C. Santrich, and D. C. Barker. 2002. PCR-based diagnosis of acute and chronic cutaneous leishmaniasis caused by *Leishmania (Viannia)*. *J. Clin. Microbiol.* **40**:601–606.
- Weigle, K. A., L. Valderrama, A. L. Arias, C. Santrich, and N. Saravia. 1991. Leishmanin skin test standardization and evaluation of safety, dose, storage, longevity of reaction and sensitization. *Am. J. Trop. Med. Hyg.* **44**:260–271.
- Wilson, E. B. 1927. Probable inference, the law of succession, and statistical inference. *J. Am. Stat. Assoc.* **22**:209–212.