Optimization of Microculture and Evaluation of Miniculture for the Isolation of *Leishmania* Parasites from Cutaneous Lesions in Peru

Andrea K. Boggild, Cesar Miranda-Verastegui, Diego Espinosa, Jorge Arevalo, Dalila Martinez-Medina, Alejandro Llanos-Cuentas, and Donald E. Low

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; Instituto de Medicina Tropical Alexander von Humboldt, and Departamento de Bioquímica, Biología Molecular y Farmacología, Facultad de Ciencias, Universidad Peruana Cayetano Heredia, Lima, Peru; Hospital Nacional Cayetano Heredia, Lima, Peru; Laboratories Branch, Ontario Ministry of Health and Long-term Care, Etobicoke, Ontario, Canada

*Address correspondence to Andrea K. Boggild, Tropical Diseases Unit, Toronto General Hospital, 200 Elizabeth Street, North Wing, 13th Floor, Room 1350, Toronto, Ontario M5G 2C4, Canada. E-mail: andrea.boggild@utoronto.ca*

**Abstract.** Traditional culture of *Leishmania* parasites is labor-intensive and shows poor sensitivity. We evaluated microculture and novel miniculture methods for diagnosis of cutaneous leishmaniasis (CL). Consecutive patients who came to the Leishmaniasis Clinic, Hospital Nacional Cayetano Heredia, Lima, Peru, were enrolled. Lesion aspirates were cultured in traditional tubes containing Novy-MacNeal-Nicolle medium and in miniculture tubes (Eppendorf, Hamburg, Germany) and capillary tubes (microculture) containing RPMI 1640 medium containing 20% fetal bovine serum. The reference standard was positive results in two of four tests (smear, culture, polymerase chain reaction, or leishmanin skin test). Outcome measures were sensitivity and time to positivity. Fifty-five patients with 74 lesions were enrolled. Of 59 lesions that fulfilled reference criteria for CL, 50 were positive by microculture (sensitivity = 84.7%; *P* = 0.001), 45 by miniculture (sensitivity = 76.3%; *P* = 0.042), and 35 by traditional culture (sensitivity = 59.3%). Median time to positivity was three days by microculture and miniculture and five days by traditional culture (*P* < 0.001). Microculture and miniculture are sensitive and efficient means of diagnosing CL.

**INTRODUCTION**

Cutaneous leishmaniasis (CL) affects up to 1.5 million people throughout the tropics and sub-tropics annually, with more than 10,000 cases occurring each year in Peru. The causative agent of CL in Peru is predominantly *Leishmania* (*Viannia* braziliensis), although other related New World species such as *L.* (*V.*) peruiana, *L.* (*V.*) guyanensis, *L.* (*V.*) lainsoni, and *L.* (*L.*) amazonensis have also been isolated. Unlike Old World CL, which does not progress to mucosal involvement, New World CL generally necessitates treatment. However, in accordance with national and international standards, treatment is rarely administered empirically in settings with diagnostic capabilities because of the toxicity associated with pentavalent antimonial therapy, underscoring the need for timely diagnosis. Definitive diagnosis of CL depends on the demonstration of parasites by direct microscopic examination of tissue aspirates, smears, or biopsy specimens, or culture of these specimens.

Nucleic acid amplification techniques such as the polymerase chain reaction (PCR) have diagnostic sensitivities on the order of 95% in acute lesions, but their practical utility in clinical and field settings is limited by their high costs, infrastructural requirements, and lack of trained personnel. Thus, culture-based testing remains a practical approach for under-resourced settings. Cultivation has the further advantage of isolating organisms for drug susceptibility testing, speciation, and genotyping, which are important in countries where infection with *L.* (*V.*) braziliensis can lead to mucosal disease, and diverse *Leishmania* species coexist, as is the case in Peru.

Traditional culture methods consist of biphasic culture systems (blood agar with a liquid overlay that is sampled periodically throughout incubation for the presence of motile promastigotes) and have sensitivities on the order of 40–75%. The microculture method consists of 70-µL capillary tubes with single-phase liquid medium supplemented with varying concentrations of fetal bovine serum (FBS). It has the advantage of being less costly because of the smaller volume of medium required and a 10-fold cost reduction between traditional culture tubes and capillary tubes, easier to use, and more sensitive, even when parasite burden is low. The higher sensitivity of microculture is thought to be related to the microaerophilic conditions and high CO2 levels established within the system, both of which are permissive to amastigote-to-promastigote transformation. One limitation of microculture is that the organism, once isolated, is relatively inaccessible because of the sealed ends of the capillary tubes. We postulated that a monophasic liquid medium in 1.5-mL Eppendorf (Hamburg, Germany) tubes (miniculture) may capture the advantages of microculture and traditional culture systems. Like capillary tubes, Eppendorf tubes are inexpensive, have a small volume, and would enable easy generation of microaerophila without a significant dilutional effect. And like traditional culture, these tubes would enable easy access to the isolate for post-culture analysis including speciation and drug susceptibility testing.

We have previously demonstrated the superiority of microculture compared with traditional culture for isolation of *Leishmania* parasites from cutaneous lesions in Peru by using RPMI 1640 medium with 10% FBS. With the objective of optimizing culture performance, we evaluated a 20% FBS-enriched microculture method and novel miniculture method by comparing them to traditional culture and other methods including leishmanin skin testing, lesion smear, and PCR for the diagnosis of CL in patients coming to a specialized leishmaniasis clinic in Lima, Peru.

**METHODS**

**Study site.** The study was conducted at the Leishmaniasis Clinic of the Instituto de Medicina Tropical Alexander Von Humboldt and the Hospital Nacional Cayetano Heredia in
Lima, Peru, from January through March 2008, following Institutional Review Board approval. The institute houses a large outpatient clinic for the diagnosis and management of CL and mucocutaneous leishmaniasis, with an average of 30–40 new cases diagnosed per month.

**Study population.** Consecutive patients presenting to the Leishmaniasis Clinic for evaluation of skin lesions were approached to participate in this study and screened for eligibility criteria. We included patients who were referred to the Leishmaniasis Clinic for suspected CL, had a clinical indication for skin scraping or aspirate, and were able to give verbal informed consent for the diagnostic procedure. We excluded patients with intercurrent bacterial or fungal superinfection of the ulcer and those undergoing active treatment of CL.

**Sampling.** Lesion aspirates and culture. Skin lesions were cleansed with topical antiseptic and aspirated in duplicate by inserting a 20-gauge needle with syringe containing 0.6 mL of sterile phosphate-buffered saline with 1,000 U/mL of penicillin and 0.3 mg/mL of streptomycin into the outer border and base of the lesion and vigorously aspirating tissue fluid as the syringe was rotated. Aspirated fluid was divided evenly in a biosafety cabinet under sterile conditions and inoculated in parallel and duplicate as follows: 1) 200 μL into 16 × 110 mm flat-sided tissue culture tubes (Nalge Nunc International, Rochester, NY) containing 3 mL modified Novy-MacNeal-Nicolle (NNN) medium (blood agar base, cat. no. 245400; Difco, Detroit, MI) with 15% defibrinated rabbit blood, 2) 200 μL into 1.5-mL sterile Eppendorf tubes containing 1.3 mL of RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, and 2 mM NaHCO3, and the pH adjusted to 7.3 (20% RPMI 1640 miniculture), or 3) 60 μL of a 1:1 mixture of aspirate and RPMI 1640 medium containing 20% FBS into sterile, non-heparinized 1 × 75 mm capillary tubes (Chase Scientific Glass, Rockwood, TN). For inoculation of capillary tubes, 200 μL of aspirate was first mixed with 200 μL of RPMI 1640 medium containing 20% FBS in a sterile Eppendorf tube. After inoculation, capillary tubes were sealed with commercially available capillary tube sealant (Fisher Scientific, Ottawa, Ontario, Canada). The remaining sample was stored at −20°C for qualitative PCR testing. Cultures were labeled with the patient’s unique identifier and date of collection, incubated horizontally at 22–26°C under standard atmospheric conditions, and examined every 1–2 days under an inverted microscope at 200× magnification. Cultures were incubated and examined for 21 days before being considered negative. Positive control cultures were inoculated as described for study specimens once a week. Uninoculated negative controls were also set up once a week.

**Smears.** Smears were obtained to quantify the amastigote burden in the lesion. After cleansing with topical antiseptic, lesion material was scraped from the ulcer base and border using a sterile lancet and spread on a glass slide. Slides were air-dried, fixed in methanol, and stained with Giemsa. Slides were examined under light microscopy and amastigotes were quantitated as per the method of Chulay and Bryceson.22 Lancets were stored at −20°C in 1.5-mL Eppendorf tubes containing 700 μL of 100% ethanol for qualitative PCR testing.

**Leishmanin skin test.** Leishmanin skin testing measures delayed-type hypersensitivity reactions to an intradermal injection of killed promastigote suspension. After cleaning the volar surface of the forearm with topical antiseptic, 0.1 mL of sterile, heat-killed promastigote lysate in 0.005% thimerosal was injected intradermally. Leishmanin skin tests were read 48 hours after administration, and a positive result was indicated by ≥ 5 mm of erythema and induration as previously described.23

**Isolation of kinetoplast DNA (kDNA) from aspirates and lancets.** Prior to DNA extraction, frozen aspirates were incubated at room temperature with 500 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) for 5 minutes to remove contaminants. Samples were then centrifuged at 8,050 × g for 10 minutes and the supernatant was discarded. Tissue traces were removed from stored lancets using sterile micropipette tips and transferred to microcentrifuge tubes. The kDNA isolation was performed with the phenol/chloroform/isoamyl alcohol method as previously described.24

**Polymerase chain reaction.** The PCR was performed using the HotStar Taq DNA Polymerase Kit (Qiagen, Lima, Peru). The final volume of the reaction mixture was 25 μL. The PCR conditions were as follows: 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 10 minutes (iCycler iQ; Bio-Rad, Hercules, CA). Two pairs of primers were used for this reaction. The first, specific for *Leishmania* subgenus *Viannia*, had the following sequences: MP1-L (fwd) 5′-TACTCCCCGACATGCTCTGTG-3′ and MP3-H (rev) 5′-GAACGGGGTTTCTGTATGC-3′, and generated a product of 70 basepairs.23 Sequences of control primers, which amplify a region of the human beta hemoglobin gene, were HBBL (fwd) 5′-GCCAGACTTCTCTCTGGAAGTCTC-3′ and HBBR (rev) 5′-CTTAGACCTCACCCTGGAGAC-3′. These primers generated a product of 197 basepairs. Amplicons were visualized after electrophoresis on 4% agarose gels (BioMOL; Promega, Lima, Peru) and stained with ethidium bromide (Figure 1).

The PCR for subgenus discrimination was then performed on all kDNA-positive samples throughout the study, and in post-hoc analysis of specimens that were composite reference positive, but kDNA PCR negative (Figure 2). The PCR was performed as above by using the following conditions: 94°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 15 seconds; extension at 72°C for 15 seconds, and a final exten-

![Figure 1](image-url) **Figure 1.** Representative agarose gel stained with ethidium bromide after polymerase chain reaction amplification of a conserved region of kinetoplast DNA specific for *Leishmania* subgenus *Viannia*. L = lancet; A = aspirate.
TABLE 1

<table>
<thead>
<tr>
<th>Assay No.</th>
<th>positive</th>
<th>negative</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNN medium</td>
<td>35</td>
<td>39</td>
<td>59.3%</td>
<td>100%</td>
<td>100%</td>
<td>38.5%</td>
</tr>
<tr>
<td>RPMI 1640 medium containing 20% FBS (miniculture)</td>
<td>45</td>
<td>29</td>
<td>76.3%</td>
<td>100%</td>
<td>100%</td>
<td>51.7%</td>
</tr>
<tr>
<td>RPMI 1640 medium containing 20% FBS (microculture)</td>
<td>50</td>
<td>24</td>
<td>84.7%</td>
<td>100%</td>
<td>100%</td>
<td>62.5%</td>
</tr>
<tr>
<td>All culture</td>
<td>50</td>
<td>24</td>
<td>84.7%</td>
<td>100%</td>
<td>100%</td>
<td>62.5%</td>
</tr>
<tr>
<td>Kinetoplast DNA PCR</td>
<td>60</td>
<td>14</td>
<td>96.6%</td>
<td>80%</td>
<td>95%</td>
<td>85.7%</td>
</tr>
</tbody>
</table>

RESULTS

Fifty-five patients (38 male and 17 female) with 74 skin lesions were enrolled in the study. Median age was 27 years (range 4–89 years) and median duration of exposure in the risk area was 24 months (range 1 week to 88 years). Work in agriculture was the principal risk occupation (31%), followed by tourists (24%). The most common lesion types were ulcers, with an almost equivalent number of lesions having a nodular or verrucous presentation (12% and 11%, respectively). Lesions were primarily located on the face (35%), extremities (32%), or lower extremities (30%).

Using the composite reference standard at least two of four test results were positive, we showed that 59 lesions were positive by at least two of four tests and 54 were positive by at least three of four tests. Sensitivity of all sensitivity of microculture was 84.7% (95% confidence interval [CI] = 75.5–93.9%) and traditional culture had a sensitivity of 59.3% (95% CI = 46.8–71.8%).

Composite reference standard. We defined a lesion as CL when the results of two of four tests (leishmanin skin test, PCR) were positive. These four tests served as the composite test was compared to all other tests.
(P = 0.001) (Table 1). Miniculture in RPMI 1640 medium containing 20% FBS had a sensitivity of 76.3% (95% CI = 62.7–84.2%) (P = 0.042 compared with traditional culture). 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: 84.8%, 78.2%, and 58.7% in microculture, RPMI 1640 medium containing 20% FBS miniculture, and NNN medium, respectively.

**Time to positivity.** Median time to culture positivity was three days by microculture (range = 2–7 days), three days by RPMI 1640 medium containing 20% FBS miniculture (range = 1–12 days), and five days by traditional culture with NNN medium (range = 3–13 days) (P < 0.001) (Table 2). When the individual patient was used as the unit of analysis, median time to culture positivity did not change significantly from the per-lesion analysis, and remained statistically significant: three days in microculture and RPMI 1640 medium containing 20% FBS miniculture, and six days in traditional culture with NNN medium. There was no discordance in positive and negative control cultures, and there was no evidence of bacterial contamination in any of the control or study specimens.

**Smears.** Thirty-nine lesions were positive by Giemsa-stained smear, yielding a sensitivity of 66.1% (95% CI = 54–78.2%) (Table 1). In those lesions that were smear positive, median smear amastigote density was grade 1 (1–10 amastigotes/100 high-power fields). In those lesions that fulfilled the composite reference standard diagnostic criteria, median smear density was higher in those that were also culture positive (density grade 2 [1–10 amastigotes/100 high-power fields]) compared with those that were culture negative (density grade 1) (P = 0.001).

**Polymerase chain reaction.** Sixty lesions were positive by PCR (Figure 1), yielding a sensitivity of 96.6% (95% CI = 92–100%) (Table 1). However, only 57 of those positive by PCR fulfilled composite reference standard diagnostic criteria. Therefore, in three lesions, PCR was the only positive test result. Compared with the composite reference standard, specificity of PCR was 80% (95% CI = 60–100%) (Table 1). Subgenus PCR was performed on all kDNA-PCR positive specimens, and post-hoc on two specimens that were kDNA negative, but composite reference standard positive. In kDNA-positive lesions, subgenus discrimination showed that all belonged to members of the *Leishmania Viannia* complex. The two lesions that were kDNA negative but composite reference standard positive were negative by subgenus PCR.

**Leishmanin skin test.** Fifty-seven lesions were from patients with positive leishmanin skin test results, yielding a sensitivity of 89.7% (95% CI = 81.9–97.5%) (Table 1). In all but three patients with a positive leishmanin skin test result, at least one other diagnostic test showed a positive result. In the one patient whose final diagnosis was biopsy-confirmed sporotrichosis, the leishmanin skin test result was negative.

Table 3 provides a comparison of sensitivity and specificity of diagnostic methods on the basis of lesion appearance. Although numbers of nodular and verrucous lesions were too small for statistical comparison, we observed that methods based on direct detection of the parasite (i.e., smear and culture) were generally less sensitive for these types of lesions.

**DISCUSSION**

We have demonstrated that an optimized microculture technique and a new miniculture method offer sensitive alternatives to biphasic, traditional culture systems. Microculture and miniculture using RPMI 1640 medium containing 20% FBS demonstrated a 43% and 29% increase in sensitivity compared with traditional culture with NNN medium, respectively. Enrichment of RPMI 1640 medium with 20% FBS resulted in an 18% improvement in microculture sensitivity compared with previous analyses in which RPMI 1640 medium containing 10% FBS was used. That microaerophilia and lower O₂:CO₂ ratios are important for amastigote-to-promastigote conversion is supported by the success of miniculture compared with RPMI 1640 medium containing 10% FBS in a traditional culture system.

In addition to improved sensitivity, time-to-culture positivity was also reduced with both microculture and miniculture, which afforded a two-day incubation savings compared with traditional NNN medium culture. However, enrichment of the microculture medium to RPMI 1640 medium containing 20% FBS did not result in any additional incubation savings over what has been previously attained with RPMI 1640 medium containing 10% FBS. Administration of systemic antimonials in the course of infection with *L. (V.) braziliensis* has been associated with a reduced risk of developing mucosal lesions. However, pentavalent antimonial therapy for New World CL is potentially toxic. Thus, treatment initiation only follows definitive diagnosis and is not administered empirically. Pentavalent antimonials including sodium stibogluconate and meglumine antimoniate are toxic to the liver, bone marrow, and heart, where induction of cardiac arrhythmias caused by QTc prolongation can occur.

Thus, highly sensitive and specific diagnostic testing with rapid turnaround time is critical for appropriate management of CL. Microculture and miniculture enable rapid isolation of the causative organism, which may facilitate more rapid species identification in centers where these capabilities exist. Species confirmation is particularly advantageous in regions where species-specific responses to pentavalent antimonials are observed.

Miniculture, although less sensitive than microculture, enables rapid cultivation of the organism, and because of easy access to the cultured parasite in Eppendorf tubes, provides a simple, economical means of propagating parasites for further analysis such as drug susceptibility testing, genomic screening.

### Table 2

<table>
<thead>
<tr>
<th>Culture method</th>
<th>Mean ± SD time to positivity, days</th>
<th>Median time to positivity, days (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microculture in RPMI 1640 medium containing 20% FBS</td>
<td>3.4 ± 0.2</td>
<td>3† (2–7)</td>
</tr>
<tr>
<td>Miniculture in RPMI 1640 medium containing 20% FBS</td>
<td>3.7 ± 0.2</td>
<td>3 (1–12)</td>
</tr>
<tr>
<td>Traditional culture in NNN medium</td>
<td>6.7 ± 0.5</td>
<td>5 (3–13)</td>
</tr>
</tbody>
</table>

* FBS = fetal bovine serum; NNN = Novy-MacNeal-Nicolle
† P < 0.001 by Kruskal-Wallis one-way analysis of variance on ranks.
Sensitivity and specificity of four diagnostic tests, on the basis of lesion appearance, used in evaluation of 74 lesions suspected to be cutaneous leishmaniasis*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ulcers (n = 57)</th>
<th>Nodular lesions (n = 9)</th>
<th>Vesicular lesions (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>LST†</td>
<td>93.0%</td>
<td>71.4%</td>
<td>75.0%</td>
</tr>
<tr>
<td>Smear</td>
<td>72.7%</td>
<td>100.0%</td>
<td>50.0%</td>
</tr>
<tr>
<td>Kinetoplast DNA PCR</td>
<td>95.5%</td>
<td>76.9%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Microculture in RPMI 1640 medium</td>
<td>88.6%</td>
<td>100.0%</td>
<td>87.5%</td>
</tr>
<tr>
<td>containing 20% FBS</td>
<td>77.3%</td>
<td>100.0%</td>
<td>87.5%</td>
</tr>
<tr>
<td>Miniculture in RPMI 1640 medium</td>
<td>70.5%</td>
<td>100.0%</td>
<td>37.5%</td>
</tr>
<tr>
<td>containing 20% FBS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional culture in NNN medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>70.5%</td>
<td>100.0%</td>
<td>37.5%</td>
</tr>
</tbody>
</table>

* LST = leishmanin skin test; PCR = polymerase chain reaction; FBS = fetal bovine serum; NNN = Novy-MacNeal-Nicolle.
† Numbers were too small for comparison of lesions stratified by appearance. One person did not undergo an LST.

or species identification by restriction fragment length polymorphism or isoenzyme analysis. The RPMI 1640 medium-based culture and propagation methods also eliminate the need for live-animal maintenance as a source for conventional blood-based Leishmania media, and the attendant risks associated with live animal work.

Giemsa-stained lesion smear, leishmanin skin testing, and kDNA PCR all had clinical sensitivities and specificities on the order of what has been reported previously. Although positive predictive values were high for all tests, indicating that positive test results are generally good predictors of disease, negative predictive values were poor for all assays, although highest with PCR. Three lesion aspirates from patients with suspected CL were positive only by kDNA and subgenus PCR, and were therefore considered negative because they failed to fulfill the composite reference criteria. These results may reflect the outperformance of PCR compared with the composite reference standard in terms of sensitivity. Because all three patients were clinically suspected to have the disease, it is possible that PCR amplified genomic material of parasites existing below the threshold of detection for the other assays. In their comparison of several PCR assays for the diagnosis of CL, Bensoussan and others similarly found that kDNA PCR had the greatest sensitivity, which led to several specimens being positive only by this test. We have similarly reported high sensitivity coupled with poor specificity of this assay when compared with a less sensitive consensus standard. Herein lies one of the major challenges of evaluating diagnostic tests in the absence of a well-performing reference standard. Although still limited by sensitivity compared with molecular methods, culture-based diagnostic testing for CL can offer a low-tech means of isolating parasites for diagnostic confirmation, species identification, and further analyses. Species confirmation remains an important diagnostic aide in regions such as Peru where multiple Leishmania species coexist and portend different prognoses. Well-documented species-specific responses to therapy in CL further underscore the clinical utility of speciation. Culture will therefore remain a necessary diagnostic complement to other assays, such as smear and skin testing, until the economic and logistical complexities of direct molecular speciation are vastly improved. We have demonstrated that enrichment of microculture with RPMI 1640 medium containing 20% FBS leads to substantial improvement in sensitivity, although affords no incubation savings over microculture in RPMI 1640 medium containing 10% FBS. Microculture has been consistently demonstrated to be more sensitive and economical, from a cost and time perspective, than traditional culture techniques used for the diagnosis of both Old World and New World species of Leishmania. Microculture has demonstrated superior sensitivity over traditional culture for isolation of L. tropica, L. infantum, L. donovani, and most recently, L. (V.) braziliensis. Our work further supports the utility of microculture for the isolation of Leishmania parasites from cutaneous lesions, and shows that RPMI 1640 medium containing 20% FBS miniculture as a slightly less-sensitive, although practical, alternative to traditional culture.

Received July 14, 2008. Accepted for publication August 27, 2008.

Acknowledgments: We thank Dr. Eduardo Gotuzzo and Ana Luz Quispe (Instituto de Medicina Tropical Alexander von Humboldt) for logistical support throughout this study.

Authors’ addresses: Andrea K. Boggild, Tropical Diseases Unit, Toronto General Hospital, 200 Elizabeth Street, North Wing, 13th Floor, Room 1350, Toronto, Ontario M5G 2C4, Canada, E-mail: andrea.boggild@utoronto.ca. Donald E. Low, Department of Microbiology, Mount Sinai Hospital, 60 University Avenue, Room 1485, Toronto, Ontario M5G 1X5, Canada, E-mail: dlow@mssinai.on.ca. Cesar Miranda-Verastegui, Diego Espinosa, Dalila Martinez-Medina, and Alejandro Llanos-Cuentas, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado #430, URB Ingenieria, Lima 31, Peru, E-mails: cesar.miranda@mail.mcgill.ca, diegoespinosa@gmail.com, dalmaria@hotmail.com, and aillanos@upch.edu.pe. Jorge Arroyo, Departamento de Bioquimica, Biologia Molecular y Farmacologia, Facultad de Ciencias, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado #430, URB Ingenieria, Lima 31, Peru, E-mail: biomoljazz@gmail.com.

REFERENCES

7. Reithinger R, Dujardin JC, Louiz H, Pirmez C, Alexander B,
11. Allahverdiyev AM, Uzun S, Bagirova M, Durdu M, Memisoglu
14. Ramírez JR, Angulo A, Muscas K, Alzate JF, Berberich C,
15. Allahverdiyev AM, Bagirova M, Uzun S, Alabaz D, Aksaray N,
17. Walsh MJ, Blum JJ, 1992. Effects of hypoxia and acute osmotic
20. Keegan F, Blum JJ, 1991. Changes in intracellular levels of fruc-
22. Chulay JD, Bryceson AD, 1983. Quantitation of amastigotes of
26. Romero GA, Vinitius de Farias Guerra M, Gomes Paes M, de
27. Tojal da Silva AC, Cupollilio E, Volpini AC, Almeida R, Sierra
diagnosis of cutaneous and mucocutaneous leishmaniasis.
29. Jones TC, Johnson WD, Barretto AC, Lago E, Badar R, Cerf B,
30. Marsden PD, 1986. Mucosal leishmaniasis (espundia Escomel,
31. Llanos-Cuentas A, Tulliano G, Araujo-Castillo R, Miranda-
32. Bensousan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL,
33. Faber WR, Oskam L, van Gool T, Kroon NC, Knecht-Junk KJ,
34. Manzur A, Bar I, 2006. Sensitivity of leishmanin skin test in
35. Weina PJ, Neafie RC, Wortmann G, Polhemus M, Aronson NE,
36. Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, Smith