*Am. J. Trop. Med. Hyg.*, 80(4), 2009, pp. 566–567


# Short Report: Evaluation of Leishmanin Skin Test in Indian Visceral Leishmaniasis

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*Abstract.* We evaluated the performance of the leishmanin skin test (LST) in 50 patients with visceral leishmaniasis (VL), 124 cured VL patients at different time intervals, 125 healthy controls from an endemic region (HEC), and 14 healthy controls from a non-endemic region (NEHC). The leishmanin antigen used was based on *Leishmania major* and was obtained from the Pasteur Institute (Iran). A positive LST was found in 14.0% of patients with active VL, 40.3% of cured patients, 21.6% of HECs, and 0% in NEHCs. The 14% positivity in patients with active VL conflicts with the widely held opinion that such patients should be negative because of anergy. Also, a lack of sensitivity of the LST was observed in cured VL patients. An LST antigen produced from *L. donovani* strains from India should be explored.

Successful cellular immune response holds the key in the control of *Leishmania* infection,[1] although the immune mechanisms by which the infection is resolved are still poorly understood. The leishmanin skin test (LST) is used as an indicator of cell-mediated immunity against leishmania parasites *invivo*. Leishmanin inoculation into the skin of an individual in whom previous leishmanial infection has led to a state of cell-mediated immunity will result in a delayed-type hypersensitivity reaction characterized by erythema and induration at the site of antigen injection after a few hours.[2]

In the areas endemic of visceral leishmaniasis (VL; or kala-azar), a significant proportion of *Leishmania*-infected individuals do not develop clinical illness but show elevated antileishmanial antibodies and/or a T-cell response to leishmanial antigens.[3,4] Although seroconversions after subclinical infections are relatively short-lived, the development of cell-mediated immunity is believed to persist for much longer and can be detected by the LST.[2] Hence, the use of the LST as a marker of previous exposure to leishmania parasites is used in many epidemiologic population-based surveys.

As a diagnostic tool, the LST has its limitations in VL, because patients with active disease are in a state of anergy and unresponsive to the LST. The LST usually becomes positive after successful drug treatment of the disease within a few weeks to months.[5] It is believed that the development of a positive LST after successful treatment correlates with long-lasting protection against leishmanial infection.[6–8] Various studies on the value of LST in clinical management of VL are often difficult to interpret because different sources of leishmanin antigen are used; they are derived from different species. At present, no standardized product is available that uses antigen derived from *L. donovani*, the causative species in India.

A valid marker of leishmanial infection is crucial for intervention studies on vaccines or vector control and for the monitoring of ongoing transmission in endemic areas. Because there was little experience with the LST in the Indian population, affected by a major *L. donovani* epidemic, we wanted to evaluate the test before its inclusion in a community intervention trial on the efficacy of bednets (see www.kalanetproject.org). In this study, we explored the value of an *L. major*–derived LST as a marker for infection and disease in Indian VL patients and endemic and non-endemic healthy controls.

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The study was designed as a "phase 1" evaluation of diagnostics and used on a limited number of purposefully selected individuals.[9] Four different groups of study subjects were recruited. 1) Patients with active VL defined as a person with fever of > 2 weeks, splenomegaly, and confirmed by parasitologic diagnosis of splenic aspirates in the clinic of the Kala-Azar Medical Research Center (KAMRC), Muzaffarpur, India. 2) Cured VL patients, i.e., a patient with previous active VL disease who received fully observed treatment of 30 days at the KAMRC treatment center in Muzaffarpur. Selection was made purposively on the basis of time elapsed since the end of drug treatment (3 and 6 months and 1, 2, 3, 4, and > 4 years), aiming for 20 patients in each subgroup. 3) Healthy endemic controls (HECs) were recruited from an endemic area in the Muzaffarpur District of Bihar state, India. Villages with several decades of past leishmanial transmission and high current prevalence of VL were selected as the study sites. 4) Non-endemic healthy controls (NEHCs) were recruited from a non-endemic area in Uttar Pradesh State.

Leishmanin antigen was obtained from the Pasteur Institute of Iran. *L. major* (reference strain MRHO/IR/75/ER) was used for preparation of the leishmanin. The preparation contained a final concentration of *L. major* promastigotes of $6 \times 10^6$ in 1 mL of phosphate-buffered saline (PBS).

The LST was performed by intradermal injection of 0.1 mL skin test antigen on the volar surface of the left forearm using a 1.0-mL sterile syringe and disposable needle. The result was read after 48–72 hours using the ballpoint pen technique.[10] Indurations were marked with a ballpoint pen, and the average of two perpendicular lines was taken as the measurement. Induration size of ≥ 5 mm was taken as a positive reading.[2]

This study was conducted at Kala-azar Medical Research Center (Infectious Diseases Research Laboratory), Banaras Hindu University, at its two sites at Varanasi, Uttar Pradesh and Muzaffarpur, Bihar, India. The study was approved by the Ethical Committee, and informed written consent was obtained from all the subjects.

The main characteristics of the 313 enrolled individuals in the four study groups are shown in Table 1. In 7 of 50 (14%)

TABLE 1
LST positivity in different subject groups

| | Patients with active VL | Cured VL patients | HEC | NEHC |
|---|---|---|---|---|
| *n* | 50 | 124 | 125 | 14 |
| LST positive (%) | 7 (14.0) | 50 (40.3) | 27 (21.0) | 0 (0.0) |

VL = visceral leishmaniasis; HEC = healthy endemic control; NEHC = non-endemic healthy control.

TABLE 2
LST in drug-cured VL patients at different time intervals after treatment

| | 3 months | 6 months | 1 year | 2 years | 3 years | 4 years | > 4 years | Total |
|---|---|---|---|---|---|---|---|---|
| *n* | 20 | 20 | 17 | 16 | 17 | 17 | 17 | 124 |
| LST positive (%) | 9 (45.0) | 5 (25.0) | 9 (53.0) | 4 (25.0) | 6 (35.3) | 7 (41.2) | 10 (58.8) | 50 (40.3) |

LST = leishmanin skin test.

patients with parasitologic confirmed active VL, the LST was positive. In patients with cured VL of varying duration (3 months [$N = 20$], 6 months [$N = 20$], 1 year [$N = 17$], 2 years [$N = 16$], 3 years [$N = 17$], 4 years [$N = 17$], and > 4 years [$N = 17$]), only 50 of 124 (40.32%) were LST positive (Table 2). On the other hand, 27 (21.6%) of 125 HECs were LST positive, and no single positive LST was observed among NEHCs. The mean diameter of LST induration was 1.8, 2.7, and 1.9 mm in active VL patients, drug-cured VL patients, and HECs, respectively (one-way ANOVA, $P = 0.066$), showing no significant difference between the groups.

The LST responses we obtained in this study were quite unexpected—in view of currently held beliefs—in the group of patients with drug-cured VL and in those with active VL. First, a significant proportion of patients with active VL had a positive LST response. Second, one would expect an increasing trend of LST positivity after drug treatment correlating with time elapsed. We found only 40.3% of the post-treatment patients were positive, with no clear trend. Manson-Bahr[11] found 80% of patients with VL developed a positive LST 6 months after successful treatment, and el Safi and others[12] found 82% were LST positive with a previous history of VL. Although most authors consider LST positivity a lifelong phenomenon, others have pointed to the phenomenon of loss of LST positivity over time, which they attributed to a loss of potency of the antigen.[13] The results we obtained in HECs and NEHCs are consistent with what one would expect in such groups, although this is hard to validate because no gold standard for previous exposure to leishmanial infection exists.

Our conclusion based on the above results was that an LST of this type could not be used as a reliable marker of previous exposure in this population, given the doubts raised about its sensitivity in persons with cured VL. If the LST antigen could be prepared using local Indian strains (*L. donovani*), this might possibly increase its sensitivity for use in the Indian subcontinent. Further to these concerns, there are other problems with the LST, such as the scarcity of good manufacturing practice grade antigen and the cumbersome reading procedure. *In vitro* T-lymphocyte tests[14,15] have been developed to document *Mycobacterium tuberculosis* infection, and a similar assay based on *Leishmania* antigen(s) might also be valuable for leishmaniasis; this might overcome some of the problems listed above. More work on the development of reliable markers of leishmanial infection is definitely needed.

Received June 12, 2008. Accepted for publication November 11, 2008.

Financial support: This project was supported by a grant from the DG Research of the European Commission (Contract INCO–CT-2005-015374, Kalanet) and the Kala-azar Medical Research Center, Sitaram Memorial Trust, Muzaffarpur, India.

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