

## Evaluation of rK-39 Strip Test Using Urine for Diagnosis of Visceral Leishmaniasis in an Endemic Region of India

Dharmendra Singh,† Krishna Pandey,† Vidya Nand Rabi Das, Sushmita Das, Neena Verma, Alok Ranjan, Sekhar Chandra Lal, Kamal Roshan Topno, Shubhankar Kumar Singh, Rakesh Bihari Verma, Ashish Kumar, Abul Hasan Sardar, Bidyut Purkait, and Pradeep Das\*

Departments of Molecular Biology, Clinical Medicine, Pathology, Biostatistics, Biochemistry, Epidemiology, and Immunology, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India

**Abstract.** The definitive diagnosis of visceral leishmaniasis (VL) requires invasive procedures for demonstration of parasites in tissue smear or culture. These procedures need expertise and laboratory supports and cannot be performed in the field. The aim of the present study was to evaluate the existing rK-39 immunochromatographic nitrocellulose strips test (ICT) with some modification in human urine for diagnosis of VL. The test was performed on both sera and urine samples on the same 786 subjects (365 confirmed VL and 421 control subjects). The sensitivity of the rK-39 ICT in serum was 100%, whereas the specificity was 93.8%, 100%, and 96.2% in healthy controls from endemic, non-endemic, and other infectious diseases, respectively. However, in urine samples, the test showed 96.1% sensitivity and 100% specificity. Considering sensitivity and feasibility of the test in the field, rK-39 ICT using urine samples can be an alternative to conventional invasive VL diagnosis.

### INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar (KA) is a vector-borne disease caused by an intracellular protozoan parasite of the *Leishmania donovani* complex, which includes *L. donovani*, *L. infantum*, and *L. chagasi*.<sup>1</sup> The clinical symptoms of VL include prolonged fever, hepatosplenomegaly, pancytopenia, hypergammaglobulinaemia, and substantial weight loss, and they are often mimicked by malaria, typhoid fever, and tuberculosis. It is 100% fatal if left undiagnosed and untreated.<sup>2</sup>

VL still remains a serious public health problem, especially in countries of the tropical, subtropical, and Mediterranean regions, where the disease is endemic.<sup>3</sup> The estimated annual global burden of VL is 200,000–400,000 new cases and more than 20,000–40,000 deaths, of which 90% occur in the Indian subcontinent (Bangladesh, India, and Nepal), Sudan, and Brazil.<sup>4</sup> In the Indian subcontinent, about 200 million people are estimated to be at risk of developing VL; this region harbors an estimated 67% of the global VL disease burden.<sup>5</sup> Bihar, an eastern Indian state, accounts for about 80% of the total burden of VL patients in India, where 33 of 38 districts are endemic.<sup>6</sup>

The confirmatory diagnosis of VL is by demonstration of leishmania parasites by Giemsa-stained smears from spleen or bone marrow and lymph node aspirates. These tests still remain the gold standard (GS) because of high specificity.<sup>7,8</sup> The aspiration materials result in a suboptimal sensitivity ranging from 93.1–98.7% for spleen aspirates to 52–85% for bone marrow and 52–58% for lymph node aspirates. However, these methods are invasive, risky, and painful; they require skilled personnel, and they are not readily available in disease-endemic regions. Moreover, VL occurs in places where health services are poorly developed.<sup>9</sup>

Several serological tests have been developed for diagnosis of VL, such as the enzyme-linked immunosorbent assay

(ELISA), the indirect immunofluorescent antibody test (IFAT), the direct agglutination test (DAT), and Western blotting.<sup>10,11</sup> These tests have relatively good sensitivities and specificities, but they have their own limitations, including cold chain requirement, skilled personnel, expensive antigen, long incubation time, sophisticated equipment, and laboratory conditions, that prohibit the application in the field.<sup>12</sup>

The use of molecular diagnostic tools, like polymerase chain reaction (PCR) and real-time PCR, is the most accurate and sensitive way to detect leishmania parasites DNA, but these tests are cumbersome to perform and have a high cost; also, they cannot be used in field conditions. In addition, well-equipped laboratories are required for their performance.<sup>13–16</sup>

Immunochromatographic nitrocellulose strips test (ICT) is simple and widely used for rapid diagnosis of VL in human blood/serum; it is extremely sensitive and specific, and it is well-accepted at the field level.<sup>17–20</sup> Because of this acceptance, rK-39 ICT has been introduced in the KA elimination program that was started in 2005 by the governments of three countries (Bangladesh, India, and Nepal) with the aim to reduce the annual incidence of VL to less than 1 case per 10,000 people at the subdistrict level by 2015 or earlier.<sup>21,22</sup> The main goal of elimination program requires early diagnostic and therapeutic tools that are very easy to use and can be easily decentralized at the periphery level.<sup>23–25</sup>

Thus, for successful control of VL from the Indian subcontinent, a simple, rapid, non-invasive, and accurate field diagnostic method is needed to improve diagnosis, especially in individuals with an increased risk of suffering complications after invasive procedures.<sup>26,27</sup> A few non-invasive sampling methods have been developed in VL diagnosis using human sputum, oral fluid, saliva, urine, and buccal swab samples with good sensitivity and specificity.<sup>28–31</sup> Advantages of the urine rK-39 ICT are that it is simple, rapid, non-invasive, and user-friendly, it has a reliable indicator, it is easy to collect, and it does not require equipment. Additionally, the results are unambiguous with high sensitivity and specificity.<sup>29,32</sup> Because young children are often vulnerable to VL infection, the use of urine will facilitate its use in field conditions.<sup>33</sup>

In this study, we evaluate the sensitivity and specificity of rK-39 ICT in diagnosis of VL using urine samples

\* Address correspondence to Pradeep Das, Department of Molecular Biology, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna—800 007, Bihar, India. E-mail: drpradeep.das@gmail.com

† These authors contributed equally.

from confirmed VL patients and controls compared with serum samples.

## MATERIALS AND METHODS

**Study design.** All VL patients enrolled in this study visited the outpatient clinic of Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, India, during the period from February of 2009 to October of 2011. These VL cases came from different endemic villages of Bihar for confirmatory diagnosis and specific treatment.

**Study subjects.** A total of 786 serum and urine samples were screened using ready to use rK-39 strips from 365 parasitologically confirmed VL patients and 421 control subjects. The control subjects were comprised of 162 healthy persons from the endemic areas who did not have a history of VL and 154 healthy persons from disease non-endemic areas, namely Agra and Mathura in Uttar Pradesh Province of India. Patients ( $N = 105$ ) with other infections, such as tuberculosis ( $N = 44$ ), malaria ( $N = 12$ ), typhoid ( $N = 10$ ), and leprosy ( $N = 39$ ), were also enrolled in this study for assessing the specificity of the urine rK-39 strip test.

**Clinical and confirmative diagnosis of VL.** Suspected VL cases with a combination of clinical signs of fever ( $> 2$  weeks duration), hepato-splenomegaly, weight loss, pancytopenia, and a positive rK-39 rapid diagnostic test for leishmania antibodies after exclusion of malaria infection (by rapid kit test) were admitted in the indoor ward for confirmative diagnosis and specific treatment. Human immunodeficiency virus (HIV) test was also performed on these patients by two rapid kits and ELISA. The confirmative diagnosis of VL was established by the demonstration of *L. donovani* (LD bodies) amastigotes in the splenic or bone marrow aspirate smears under the microscope.<sup>34</sup> Because of ethical reasons, splenic or bone marrow aspiration could not be performed in control subjects.

**Sample collection and rK-39 strip test.** Peripheral blood and urine samples were collected from all participants in the study in vacutainers by puncturing anticubital veins using a 22-gauge intravenous needle and 50-mL falcon tubes, respectively. Both blood and urine samples were stored immediately in an ice box and carried to laboratory. Blood sample was centrifuged for separation of serum at the laboratory, where the rK-39 strip test was performed and interpreted according to the manufacturer's instructions. The rK-39 strip (Kala-azar Detect; InBios International, Seattle, WA) is an immunochromatographic nitrocellulose impregnated with recombinant K-39 antigen.<sup>35</sup> For the urine assay, 200  $\mu$ L urine and 200  $\mu$ L 10% sodium azide (equal vol/vol) solution were mixed in a fresh 1.5-mL Eppendorf tube. The rK-39 strip was dipped in the mixed urine sample (1.5-mL Eppendorf tube) without adding chase buffer, and the mixture was allowed to migrate up the strip by capillary action. After 10 minutes, the appearance of a red upper (control) band indicated the presence of immunoglobulin G (IgG) and proper test functioning; a red lower (test) band indicated the presence of anti-rK-39 IgG and a positive test result. The test was considered positive when both bands (control and test) appeared within 10 minutes and negative when only the upper control band appeared.<sup>18</sup> A faint band was considered positive, whereas only a lower test band was considered an invalid test. Both

blood and urine samples were collected during the same visit, and they were blinded and tested independently.

### Inclusion and exclusion criteria.

#### Inclusion criteria.

- (1) Patients giving informed consent.
- (2) Patients between 2 and 65 years and of both sexes.
- (3) HIV-negative patients.

#### Exclusion criteria.

- (1) Pregnant and breastfeeding females.
- (2) Coinfection with HIV-positive patients.
- (3) Patients with past history of VL and treated with anti-leishmanial drugs.
- (4) Patients not willing to participate in the study.
- (5) Patients not giving voluntary informed consent.

**Chemotherapy.** After clinical and confirmatory diagnosis, all VL patients were admitted in the indoor ward and treated with antileishmanial drugs as per the protocol of different clinical trials for their respective treatment regimens. Amphotericin B (AmB; Fungizone, Abbott, Mumbai, Maharashtra, India) was administered in the dose of 1 mg/kg body weight in 5% dextrose intravenously slowly over 4–6 hours for 15 injections on alternate days.<sup>36</sup> Those patients who could not be given amphotericin B were treated with capsules of miltefosine for 28 days in the dose of 2.5 mg/kg body weight in two divided doses orally, except females of child-bearing age and pregnant females.<sup>37</sup>

**Statistical analysis.** All data were entered in a computer using Excel (Version MS Office 7). Sensitivity and specificity were computed along with 95% confidence intervals (CIs) using Stata software (version 10.0).

**Ethical approval.** The study was approved by the Institutional Ethical Committee, and written informed consent was obtained from all participants or their legal guardian before inclusion in the study.

## RESULTS

The sensitivity and specificity of the rK-39 strip test for the diagnosis of VL were evaluated with serum and urine. A total of 786 serum and urine samples from 365 parasitologically confirmed VL and 421 control subjects was tested by rK-39 strips. The serum rK-39 strip test was positive in all 365 confirmed VL subjects, whereas all 154 healthy controls from the non-endemic areas tested negative. However, 10 of 162 healthy endemic and 4 of 105 other infectious disease controls tested positive by serum rK-39. The urine rK-39 strip test was positive in 351 of 365 confirmed VL subjects, whereas all healthy controls from endemic, non-endemic areas, and other infectious diseases tested negative. Thus, the sensitivity and specificity of urine rK-39 strips were found to be 96.1% (95% CI = 93.6–97.8) and 100% (95% CI = 97–100), respectively (Table 1).  $\kappa$ -coefficients for both serum and urine rK-39 strip tests were found to be 0.93, showing a high level of agreement.

## DISCUSSION

In clinical practice as well as the ongoing KA elimination program, the rK-39 strips test has been strongly recommended on serum or finger-prick blood for rapid diagnosis of VL, but it is invasive and requires phlebotomists and sterilized needles.<sup>26</sup> There is also great difficulty in sampling blood from children and healthy persons in the field because of the high proportion of refusals. Nearly one-half of VL cases occur

TABLE 1

Comparison of sensitivity and specificity of rK-39 strip tests in diagnosis of KA using serum versus urine samples from LD body-positive VL patients and controls

Subjects	Subjects ( <i>n</i> )	rK-39 strip test results					
		Serum			Urine		
		Sensitivity <i>N</i> (%)	Specificity <i>N</i> (%)	95% CI	Sensitivity <i>N</i> (%)	Specificity <i>N</i> (%)	95% CI
Confirmed VL patients (for LD bodies in SA or BMA)	365	365 (100)	NA	98.9–100	351 (96.1)	NA	93.6–97.8
Healthy controls from disease- endemic areas	162	NA	152 (93.8)	88.9–97.0	NA	162 (100)	97.7–100
Healthy controls from disease non-endemic areas	154	NA	154 (100)	97.6–100	NA	154 (100)	97.6–100
Control patients with other infections	105	NA	101 (96.2)	90.5–98.9	NA	105 (100)	96.5–100
Total	786						

Other infections included tuberculosis (*N* = 44), malaria (*N* = 12), typhoid (*N* = 10), and leprosy (*N* = 39). BMA = bone marrow aspirate; NA = not applicable; SA = splenic aspirate.

in children.<sup>33</sup> However, collecting urine from this cohort is relatively easier because of less reluctance. Moreover, blood-borne transmission of HIV, hepatitis B surface antigen (HbsAg), and hepatitis C virus (HCV) can also occur by drawing blood through unsterile needles/syringes as well as using improper techniques.

The detection of antileishmania IgG antibodies and DNA in urine specimens for diagnosis of VL has already been developed.<sup>13,29,32,38</sup> The works by Brandtzaeg and others<sup>39</sup> and Roitt and Lehner<sup>40</sup> reported that the mean IgG concentration was 14,730 mg/L in serum and 12,500 mg/L in urine. Although several different serological test formats exist for diagnosis of VL, many of them have not been validated in prospective field studies.<sup>11,12</sup> KAtex, a latex agglutination test based on the detection of a low-molecular weight (5–20 kDa), heat-stable carbohydrate antigen from leishmania in the urine of VL patients, has close to 100% specificity but less sensitivity, and pre-treatment of urine (boiling to 100°C for 5 minutes) is required before testing, which may not be feasible in the field setting.<sup>41</sup> Here, we evaluated the usefulness of urine rK-39 ICT for diagnosis of VL and compared the results with serum. In our study, urine rK-39 ICT showed 96.1% (95% CI = 93.6–97.8) sensitivity; this finding corroborates well with the results of two other studies reported from India and Bangladesh, which showed sensitivity of 96.4% (95% CI = 94–99) and 95.0% (95% CI = 88.2–98.1), respectively. Both of these studies were performed in the same geographical region on parasitologically (LD bodies) and serologically (rK-39 test) positive VL cases, respectively.<sup>29,32</sup> In our study, the serum rK-39 ICT showed 100% sensitivity, whereas specificity was variable in control subjects. It showed that none of the healthy non-endemic controls were positive, whereas 6.2% and 3.85% of healthy endemic and other infectious disease controls gave positive reactions, respectively, which indicates false-positive reactions. These results corroborated well with the finding of other studies that reported results from the Indian subcontinent, East Africa, and Brazil.<sup>42,43</sup> However, all the false-positive serum samples by rK-39 strip from endemic and other infectious diseases were also tested by blood PCR using leishmania-specific primers from the kinetoplast DNA minicircle.<sup>15</sup> They were all negative, whereas 10 randomly chosen confirmed VL cases remained positive by blood PCR.

However, our results on the specificity of the urine rK-39 are consistent with the previous study reported in Bangladesh on healthy controls from endemic and non-endemic areas.<sup>29</sup> However, the same study showed 5 positive samples among 25 samples of malaria patients, whereas in the present study, none of the malaria samples were found to be positive,

because the sample size in this category was relatively small. Furthermore, this work needs to be corroborated with a large sample size in malaria and typhoid patients, where the symptoms are very similar to the symptoms of KA and can lead to diagnostic difficulty in clinical practice.<sup>2</sup> In a recently published Indian study, some cross-reactivity was reported with control subjects, including endemic and persons having other diseases.<sup>32</sup> Sodium azide (NaN<sub>3</sub>) is commonly used as a preservative for samples and stock solutions in laboratories to prevent microbial contamination. In our study, we observed that the use of NaN<sub>3</sub> in urine samples increased the specificity of rK-39 ICT by narrowing non-specific bindings. A report from Bangladesh also revealed no cross-reactivity with endemic controls with the use of the NaN<sub>3</sub> inhibitor.<sup>29</sup> In the present study, we used increasing concentrations of sodium azide until inhibition was accomplished, which was not specified in the other published study. We also tested other inhibitors to eliminate non-specific reactivity, such as sodium dodecyl sulphate, trichloroacetic acid, dithiothreitol, bovine serum albumin, skimmed milk, etc., but sodium azide proved to be the best among them.<sup>44</sup> All VL patients were treated with antileishmanial drugs (amphotericin B or miltefosine) and responded well, and there were no relapses.

## CONCLUSION

GS for diagnosis of VL is invasive, hazardous, time-consuming, technically demanding, and very difficult to perform in field conditions or remote areas/primary health centers. Urine rK-39 ICT has the advantages of being safe, non-invasive, and easy to collect and perform in difficult field settings. It also can be carried out in large numbers of samples, which can prove to be a boon, especially for the diagnosis of VL in the field, and it can be used as an alternative method in the KA elimination program.

Received August 10, 2012. Accepted for publication October 6, 2012.

Published online November 13, 2012.

Acknowledgments: The authors thank the Department of Health Research (DHR), Ministry of Health and Family Welfare, Government of India, for selecting this study as a top-priority program under translational research for the elimination effort of VL from India. The authors thank Mr. Manohar Kumar (laboratory assistant) and Mr. Naresh Kumar Sinha (technical assistant) for their technical and field support in the work.

Authors' addresses: Dharmendra Singh, Sushmita Das, Ashish Kumar, Abul Hasan Sardar, Bidyut Purkait, and Pradeep Das, Department of Molecular Biology, Rajendra Memorial Research Institute of Medical



Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mails: singhd72@yahoo.co.in, sushmita.de2008@gmail.com, ashish2k8@gmail.com, abulhasansardar82@gmail.com, bidybiotech@gmail.com, and rmrims@icmr.org.in. Krishna Pandey, Vidya Nand Rabi Das, and Rakesh Bihari Verma, Department of Clinical Medicine, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mails: drkrishnapandey@yahoo.com, drvnrdas@yahoo.com, and rbihariverma@yahoo.com. Neena Verma, Department of Pathology, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mail: verma\_neena@yahoo.com. Alok Ranjan, Department of Health Research Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mail: aranjan30@yahoo.com. Sekhar Chandra Lal, Department of Biochemistry, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mail: drcsral@sify.com. Kamal Roshan Topno, Department of Epidemiology, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mail: roshanktopno@yahoo.co.in. Shubhankar Kumar Singh, Immunology Division, Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Agamkuan, Patna, Bihar, India, E-mail: sksmri@yahoo.com.

## REFERENCES

- Lukes J, Mauricio IL, Schönian G, Dujardin JC, Soteriadou K, Dedet JP, Kuhls K, Tintaya KW, Jirků M, Chocholová E, Haralambous C, Pratloug F, Oborník M, Horák A, Ayala FJ, Miles MA, 2007. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Natl Acad Sci USA* 104: 9375–9380.
- Berman JD, 1997. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin Infect Dis* 24: 684–703.
- Desjeux P, 2004. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 27: 305–318.
- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M; the WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7: e35671.
- Stauch A, Sarkar RR, Picado A, Ostyn B, Sundar S, Rijal S, Boelaert M, Dujardin JC, Duerr HP, 2011. Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. *PLoS Negl Trop Dis* 5: e1405.
- Das P, Samuels S, Desjeux P, Mittal A, Topno R, Siddiqui NA, Sur D, Pandey A, Sarnoff R, 2010. Annual incidence of visceral leishmaniasis in an endemic area of Bihar, India. *Trop Med Int Health* 15 (Suppl 2): 4–11.
- Srividya G, Kulshrestha A, Singh R, Salotra P, 2012. Diagnosis of visceral leishmaniasis: developments over the last decade. *Parasitol Res* 110: 1065–1078.
- Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, Kager PA, 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans R Soc Trop Med Hyg* 86: 505–507.
- Desjeux P, 1996. Leishmaniasis. Public health aspects and control. *Clin Dermatol* 14: 417–423.
- Kumar S, Kumar D, Chakravarty J, Rai M, Sundar S, 2012. Identification and characterization of a novel *Leishmania donovani* antigen for serodiagnosis of visceral leishmaniasis. *Am J Trop Med Hyg* 86: 601–605.
- Pedras MJ, de Gouvêa Viana L, de Oliveira EJ, Rabello A, 2008. Comparative evaluation of direct agglutination test, rK39 and soluble antigen ELISA and IFAT for the diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 102: 172–178.
- Gidwani K, Kumar R, Rai M, Sundar S, 2009. Longitudinal seroepidemiologic study of visceral leishmaniasis in hyperendemic regions of Bihar, India. *Am J Trop Med Hyg* 80: 345–346.
- Fisa R, Riera C, López-Chejade P, Molina I, Gállego M, Falcó V, Ribera E, Portús M, 2008. *Leishmania infantum* DNA detection in urine from patients with visceral leishmaniasis and after treatment control. *Am J Trop Med Hyg* 78: 741–744.
- Mary C, Faraut F, Lascombe L, Dumon H, 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *J Clin Microbiol* 42: 5249–5255.
- Salotra P, Sreenivas G, Pogue GP, Lee N, Nakhasi HL, Ramesh V, Negi NS, 2001. Development of a species-specific PCR assay for detection of *Leishmania donovani* in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. *J Clin Microbiol* 39: 849–854.
- Srivastava P, Mehrotra S, Tiwary P, Chakravarty J, Sundar S, 2011. Diagnosis of Indian visceral leishmaniasis by nucleic acid detection using PCR. *PLoS One* 6: e19304.
- de Assis TS, Braga AS, Pedras MJ, Oliveira E, Barral A, de Siqueira IC, Costa CH, Costa DL, Holanda TA, Soares VY, Biá M, Caldas Ade J, Romero GA, Rabello A, 2010. Multi-centric prospective evaluation of rK39 rapid test and direct agglutination test for the diagnosis of visceral leishmaniasis in Brazil. *Trans R Soc Trop Med Hyg* 105: 81–85.
- Maia Z, Lírio M, Mistro S, Mendes CM, Mehta SR, Badaro R, 2012. Comparative study of rK39 *Leishmania* antigen for serodiagnosis of visceral leishmaniasis: systematic review with meta-analysis. *PLoS Negl Trop Dis* 6: e1484.
- Sundar S, Reed SG, Singh VP, Kumar PC, Murray HW, 1998. Rapid accurate field diagnosis of Indian visceral leishmaniasis. *Lancet* 351: 563–565.
- Zijlstra EE, Nur Y, Desjeux P, Khalil EA, El Hassan AM, Groen J, 2001. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. *Trop Med Int Health* 6: 108–113.
- Mondal D, Singh SP, Kumar N, Joshi A, Sundar S, Das P, Siddhivinayak H, Kroeger A, Boelaert M, 2009. Visceral leishmaniasis elimination program in India, Bangladesh, and Nepal: reshaping the case finding/case management strategy. *PLoS Negl Trop Dis* 3: e355.
- WHO/SEARO, 2005. *Regional Strategic Framework for Elimination of Kala-Azar from the South-East Asia Region (2005–2015)*. Geneva: WHO/SEARO.
- Bhattacharya SK, Sur D, Sinha PK, Karbwang J, 2006. Elimination of leishmaniasis (kala-azar) from the Indian subcontinent is technically feasible and operationally achievable. *Indian J Med Res* 123: 195–196.
- Hirve S, Singh SP, Kumar N, Banjara MR, Das P, Sundar S, Rijal S, Joshi A, Kroeger A, Varghese B, Thakur CP, Huda MM, Mondal D, 2010. Effectiveness and feasibility of active and passive case detection in the visceral leishmaniasis elimination initiative in India, Bangladesh, and Nepal. *Am J Trop Med Hyg* 83: 507–511.
- Thakur CP, Meenakshi Thakur AK, Thakur S, 2009. Newer strategies for the kala-azar elimination programme in India. *Indian J Med Res* 129: 102–104.
- Matlashewski G, Arana B, Kroeger A, Battacharya S, Sundar S, Das P, Sinha PK, Rijal S, Mondal D, Zilberstein D, Alvar J, 2011. Visceral leishmaniasis: elimination with existing interventions. *Lancet Infect Dis* 11: 322–325.
- Saha S, Goswami R, Pramanik N, Guha SK, Saha B, Rahman M, Mallick S, Modak D, Silva FO, Mendonca IL, Costa DL, Costa CH, Ali N, 2011. Easy test for visceral leishmaniasis and post-kala-azar dermal leishmaniasis. *Emerg Infect Dis* 17: 1304–1306.
- Galai Y, Chabchoub N, Ben-Abid M, Ben-Abda I, Ben-Alaya-Bouaif N, Amri F, Aoun K, Bouratbine A, 2011. Diagnosis of Mediterranean visceral leishmaniasis by detection of *Leishmania* antibodies and *Leishmania* DNA in oral fluid samples collected using an Oracol device. *J Clin Microbiol* 49: 3150–3153.
- Khan MG, Alam MS, Podder MP, Itoh M, Jamil KM, Haque R, Wagatsuma Y, 2010. Evaluation of rK-39 strip test using urine for diagnosis of visceral leishmaniasis in an endemic area in Bangladesh. *Parasit Vectors* 3: 114.
- Singh D, Pandey K, Das VN, Das S, Kumar S, Topno RK, Das P, 2009. Novel noninvasive method for diagnosis of visceral leishmaniasis by rK39 testing of sputum samples. *J Clin Microbiol* 47: 2684–2685.
- Vaish M, Singh OP, Chakravarty J, Sundar S, 2012. rK39 antigen for the diagnosis of visceral leishmaniasis by using human saliva. *Am J Trop Med Hyg* 86: 598–600.
- Chakravarty J, Kumar S, Kumar R, Gautam S, Rai M, Sundar S, 2011. Evaluation of rK39 immunochromatographic test with urine for diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 105: 537–539.

33. Cruz I, Chicharro C, Nieto J, Bailo B, Cañavate C, Figueras MC, Alvar J, 2006. Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. *J Clin Microbiol* 44: 2343–2347.
34. Chulay JD, Bryceson AD, 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.
35. Badaró R, Benson D, Eulálio MC, Freire M, Cunha S, Netto EM, Pedral-Sampaio D, Madureira C, Burns JM, Houghton RL, David JR, Reed SG, 1996. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *J Infect Dis* 173: 758–761.
36. Thakur CP, Ahmed S, 2001. Observations on amphotericin B treatment of kala-azar given in a rural set up in Bihar, India. *Indian J Med Res* 113: 14–18.
37. Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, Boelaert M, Dujardin JC, Chakravarty J, 2012. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis* 55: 543–550.
38. Solano-Gallego L, Rodríguez A, Iniesta L, Arboix M, Portús M, Alberola J, 2003. Detection of anti-*Leishmania* immunoglobulin G antibodies in urine specimens of dogs with leishmaniasis. *Clin Diagn Lab Immunol* 10: 849–855.
39. Brandtzaeg P, Fjellanger I, Gjeruldsen ST, 1970. Human secretory immunoglobulins. I. Salivary secretions from individuals with normal or low levels of serum immunoglobulins. *Scand J Haematol Suppl* 12: 3–83.
40. Roitt I, Lehner T, 1983. *Immunology of Oral Diseases, 2nd edition*. Oxford: Blackwell Scientific Publications, 279–280.
41. Ghatei MA, Hatam GR, Hossini MH, Sarkari B, 2009. Performance of latex agglutination test (KAtex) in diagnosis of visceral leishmaniasis in Iran. *Iran J Immunol* 6: 202–207.
42. Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, Wasunna M, Aseffa A, Mbui J, Menten J, Desjeux P, Peeling RW, 2008. Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. *Trans R Soc Trop Med Hyg* 102: 32–40.
43. Carvalho SF, Lemos EM, Corey R, Dietze R, 2003. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *Am J Trop Med Hyg* 68: 321–324.
44. Hatam GR, Ghatee MA, Hossini SM, Sarkari B, 2009. Improvement of the newly developed latex agglutination test (Katex) for diagnosis of visceral leishmaniasis. *J Clin Lab Anal* 23: 202–205.