Clinical Infectious Diseases

BRIEF REPORT

A Novel Antigen, Otubain Cysteine Peptidase of *Leishmania donovani*, for the Serodiagnosis of Visceral Leishmaniasis and for Monitoring Treatment Response

Mohd Kamran,[1,a] Sarfaraz Ahmad Ejazi,[1,a] Somsubhra Thakur Choudhury,[1,b] Anirban Bhattacharyya,[1] Km Tanishka,[1,c] Krishna Pandey,[2] Vidya Nand Ravi Das,[2] Pradeep Das,[2] Fernando Oliveira da Silva,[3] Dorcas Lamounier Costa,[3] Carlos Henrique Nery Costa,[3] Mehebubar Rahaman,[4] Rama Prosad Goswami,[4] and Nahid Ali[1]

[1]Infectious Diseases and Immunology Division, Council of Scientific and Industrial Research–Indian Institute of Chemical Biology, Kolkata, India; [2]Rajendra Memorial Research Institute of Medical Sciences, Patna, India; [3]Universidade Federal do Piaui, Teresina, Brazil; and [4]Department of Tropical Medicine, School of Tropical Medicine, Kolkata, India

Tests for visceral leishmaniasis (VL) are not uniformly effective for all endemic regions. In a serological assay, a novel antigen, otubain cysteine peptidase, compared with rK39, showed comparable sensitivity with Indian VL serum samples and prominently increased sensitivity with Brazilian samples, as well as improved monitoring of the treatment response.

Keywords. Leishmaniasis; diagnosis; serology; rK39; lateral flow assay.

Visceral leishmaniasis (VL) or kala-azar, a potentially devastating parasitic disease is currently endemic in >60 countries, with annual new cases of 200 000–400 000 globally [1]. Microscopic observation of the parasites in splenic and bone marrow aspirates remains the reference standard test for VL diagnosis. Parasites in skin lesions of post–kala-azar dermal leishmaniasis (PKDL), an outcome of VL, are detected by means of slit-skin smear. However, the invasiveness of these methods and the technical expertise they require has prompted the development of easy-to-use serological tests, such as direct agglutination tests, enzyme-linked immunosorbent assays (ELISAs), and rapid diagnostic tests [2].

Rapid diagnostic tests based on the rK39 antigen are widely used for routine diagnosis of VL, particularly in the Indian subcontinent. However, their effectiveness is lower in other regions of VL endemicity, such as Brazil and Sudan. Because *Leishmania*-specific immunoglobulin (Ig) G antibodies remain in the blood long after treatment, serological tests cannot distinguish between active and past infections [3]. Most of the recombinant antigens tested during the last decade, such as rKE16, rK28, and rKLO8, are from the kinesin-related family, like rk39, with variable sensitivities and specificities [4]. To improve early diagnosis and treatment response, there is a vital need to identify new biomarkers that have greater sensitivity and specificity for all endemic regions and ideally should be a test of cure.

Deubiquitinases are cysteine peptidases that remove ubiquitin from substrate tagged for proteasomal degradation [5]. In recent years, 20 deubiquitinases in 7 families, including otubains, have been identified in *Leishmania,* and their essential role in parasite life cycle progression has been reported [6]. Based on sequence homology, *Leishmania donovani*–otubain cysteine peptidase (Ld-OCP) was selected among other OCPs and was least similar in amino sequence to human otubain-1 (33%) and otubain-2 (26%) and high conservation with subgenus *Leishmania infantum* (99%) [7].

In the current study, we cloned and isolated a novel recombinant antigen, Ld-OCP, from *L. donovani* promastigotes. After validating its diagnostic ability with ELISA and immunoblot assay, we developed a lateral flow–based rapid diagnostic test and compared it with the commercially available rK39 strip test.

PATIENTS, MATERIALS, AND METHODS

A total of 186 serum samples from India and Brazil were used for this study. Serum samples from 65 parasitologically confirmed VL cases, 15 cases after 6 months of treatment (VL follow-up) and 6 PKDL cases were collected from the School of Tropical Medicine, Kolkata, India, and Rajendra Memorial Research Institute of Medical Sciences, Patna, India, between 2017 and 2020. Serum samples from 39 patients with parasitologically proved VL and 22 healthy controls were also obtained from Universidade Federal do Piaui, Teresina, Brazil; these samples were collected between 2008 and 2009 and transported to India under frozen conditions and kept at −80°C until use. This study was approved by the Ethical Committee of Indian Institute of Chemical Biology, Kolkata, India, and written informed consent was obtained from each participant.

The Ld-OCP gene from *L. donovani* (American Type Culture Collection PRA-413) was cloned, and purified protein was obtained (Supplementary Figure 2*A*). ELISA and immunoblot assay were performed with antigen Ld-OCP, as described elsewhere [8]. For the lateral flow assay (LFA), nitrocellulose membrane (0.45 µm; mdi Membrane Technologies) was used to coat

Received 11 February 2021; editorial decision 4 May 2021; published online 14 May 2021.

[a]M. K. and S. A. E. contributed equally to this work.

[b]Present affiliation: Department of Biotechnology, Sister Nivedita University, West Bengal, India.

[c]Present affiliation: Department of Biochemistry, University of Hyderabad, India.

Correspondence: Nahid Ali, Infectious Diseases and Immunology Division, Council of Scientific and Industrial Research-Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Jadavpur, Kolkata 700032, India (nali@iicb.res.in).

Clinical Infectious Diseases® 2021;73(7):1281–3

DOI: 10.1093/cid/ciab435

the antigen, Ld-OCP (1 mg/mL), and anti-mouse antibody (1 mg/mL) at the test and control lines, respectively, using an automatic dispenser (Advanced Sensor Systems).

Coated membranes were dried at 37°C for 30 minutes. The conjugate pad was coated with protein G–conjugated colloidal gold (Ubio Biotechnology Systems) in 10% sucrose buffer and dried at 37°C for 30 minutes. Coated membranes were assembled with the substrate and absorbent pads according to the standard protocol and were cut into 2-mm-thick strips. Strips were fixed in the cassette and stored under desiccated conditions. For the LFA, 10 µL of serum was applied at the sample pad, followed by 25 µL of chase buffer (1% bovine serum albumin with 0.1% Tween 20, 9% sodium chloride, and 0.1% sodium azide). The result became visible as colored bands within 2–5 minutes. rK39 strip tests (Kalazar Detect Rapid Test; InBios International) were conducted according to the manufacturer's protocol.

Statistical analysis was performed with GraphPad Prism software, version 7.0. Mann-Whitney *U* tests were used, and differences were considered statistically significant at $P < .05$. The sensitivity and specificity for ELISA were determined based on the cutoff obtained from receiver operating characteristic curves.

RESULTS

ELISA performed with Ld-OCP with Indian serum samples had 96.92% sensitivity and 97.43% specificity (Supplementary Figure 1*A*). Two samples from VL-positive cases were found to be false-negative, whereas 1 healthy control showed cross-reactivity with the antigen. Ld-OCP ELISA with Brazilian serum samples had 97.43% sensitivity and 100% specificity. Of the 39 serum samples from active VL cases, only 1 had a false-negative result. No healthy control sample had a false-positive result (Supplementary Figure 1*B*).

A total of 9 Indian VL samples were tested with the immunoblot assay. All of the active VL serum samples recognized the Ld-OCP at 31.5 kDa (Supplementary Figure 2*B*). Control serum samples, however, did not show cross-reactivity with the Ld-OCP; these included 2 samples from healthy controls in nonendemic regions, 2 from healthy controls in endemic regions, and 1 each from patients with malaria, tuberculosis, viral fever, or typhoid (Supplementary Figure 2C).

Next, we performed Ld-OCP–based LFA, comparing it with the commercially available rK39 strip test, using 49 confirmed Indian VL samples, 15 follow-up VL samples, 6 PKDL samples, 18 samples from healthy controls in nonendemic regions, 7 from healthy controls in endemic regions, and 10 from patients with other diseases. All VL-positive samples showed distinct test and control bands with Ld-OCP LFA, suggesting 100% sensitivity, similar to results with rK39. Of 6 PKDL cases tested, Ld-OCP showed absolute reactivity, unlike rK39, which had 1 false-negative result. Only 1 sample from a healthy control in an endemic region showed false-positivity, resulting in 97.14% specificity for our LFA. Interestingly, follow-up samples after 6 months of VL treatment did not show reactivity with Ld-OCP LFA, whereas 86.66% of follow-up samples were still positive with the rK39 strip test. This suggests an excellent prognostic potential for Ld-OCP in India.

Similarly, Ld-OCP–based LFA and its comparative study with rK39 were performed with Brazilian serum samples, using 39 confirmed VL samples and 20 from healthy controls. Ld-OCP LFA demonstrated 100% sensitivity with Brazilian VL samples, compared with the rK39 test, which showed a low sensitivity of only 64.10%. The specificity of Ld-OCP with Brazilian healthy controls was found to be 90%, compared with 100% for the rK39 test. A representative Ld-OCP LFA is shown in Supplementary Figure 3. Results for both assays, ELISA and LFA, are summarized in Table 1.

Table 1. Performance of *Leishmania donovani*–Otubain Cysteine Peptidase in the Serodiagnosis of Indian and Brazilian Visceral Leishmaniasis Cases

Country and Test	Antigen Used	Serum Samples, No./Total					
		VL (Sensitivity, %)	VL Follow-up (Reactivity, %)	NEHC (Specificity, %)	EHC (Specificity, %)	OD (Specificity, %)	Total Controls (Specificity, %)
India							
ELISA	Ld-OCP	63/65 (96.92); PKDL: 6/6 (100)	0/15 (0)	19/20 (95)	7/7 (100)	12/12 (100)	38/39 (97.43)
LFA	Ld-OCP	49/49 (100); PKDL: 6/6 (100)	0/15 (0)	18/18 (100)	6/7 (85.71)	10/10 (100)	34/35 (97.14)
	rK39	49/49 (100); PKDL: 5/6 (83.33)	13/15 (86.66)	18/18 (100)	7/7 (100)	10/10 (100)	35/35 (100)
Brazil							
ELISA	Ld-OCP	38/39 (97.43)	…	22/22 (100)	…	…	22/22 (100)
LFA	Ld-OCP	39/39 (100)	…	18/20 (90)	…	…	18/20 (90)
	rK39	25/39 (64.10)	…	20/20 (100)	…	…	20/20 (100)

Abbreviations: EHC, healthy controls from endemic areas; NEHC, healthy controls from nonendemic areas; OD; other diseases; ELISA, enzyme-linked immunosorbent assay; Ld-OCP, *Leishmania donovani*–otubain cysteine peptidase; LFA, lateral flow assay; NEHC, ; OD, ; PKDL, post–kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.

DISCUSSION

In the last 2 decades many leishmanial antigens, lysates, and recombinants were developed and used to detect total IgG in serological assays for VL diagnosis [4, 9]. However, total IgG levels that remain elevated even after treatment led to the failure of the current serological tests for monitoring treatment [10]. Moreover, there is no single antigen available for VL detection that performed well in all areas of endemicity.

These observations warranted the quest for novel antigenic tests with reliable performance in all areas of VL endemicity, which should ideally have prognostic potential. In the current study, we explored the diagnostic potential of a novel antigen, Ld-OCP, which showed excellent performance with Indian as well as Brazilian VL serum samples. In addition, this test overcomes the current problem with timely assessment of treatment response, by showing completely negative reactivity after 6 months of VL treatment.

Among previously described antigens, rk39-based diagnostic tests are currently being used worldwide, despite their low sensitivity in South America and parts of East Africa [11]. Although rk39 shows good sensitivity (>95%) in the Indian subcontinent, earlier reports suggested its cross-reactivity with samples from some healthy controls in VL-endemic regions [12]. Ld-OCP in ELISA exhibited 96.92% sensitivity and 97.43% specificity with Indian serum samples, versus 97.43% sensitivity and 100% specificity from Brazilian samples. Moreover, Ld-OCP in ELISA distinguished active Indian VL cases from cases after 6 months of treatment, with 100% specificity, suggesting a prognostic potential for this antigen.

In the LFA format, Ld-OCP showed high sensitivity for diagnosing VL in Indian patients. Furthermore, the test registered no reactivity (0%) with serum samples obtained 6 months after VL treatment, validating the antigen's prognostic potential. Thus, Ld-OCP demonstrates a positive advantage over the rK39 test, which retained 86.66% reactivity "after 6 months treatment". Furthermore, contrary to the low sensitivity of rk39 (64.10%) in detecting Brazilian VL cases, Ld-OCP LFA showed 100% sensitivity, indicating excellent VL detection ability in Brazil.

Although the current results are encouraging, our study has certain limitations. The VL cases used were clinically confirmed cases; thus, we cannot predict VL positivity among suspected cases. Further evaluation of our test in phase III validation trials will reveal its true performance under field conditions. Moreover, our study lacks some control groups in Brazil. Future validation studies in Brazil will be conducted to ensure the test's specificity against other diseases, such as cutaneous leishmaniasis and Chagas disease.

Within the existing limitations, the Ld-OCP LFA has shown promising results in this preliminary study, for use in rapid diagnosis of VL and to monitor treatment response. Therefore, the test qualifies for further studies in the next phase of the work, using more samples within defined groups of VL cases and controls in several endemic areas.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank Clayton Fernando, Puja Kumari, and Subhadeep Das for their help in performing the experiments. We also thank Anand Kumar Gupta, Sonali Das, Sneha Ghosh, and Nicky Didwania for their help in proofreading the manuscript.

Financial support. This work was supported by UK Research and Innovation via the Global Challenges Research Fund (grant agreement "A Global Network for Neglected Tropical Diseases"; grant MR/P027989/1), the Sir J. C. Bose Fellowship, Fast Track Translational Research Project (33/BS/FTT/2016-MD) of the Council of Scientific and Industrial Research, India, and the Indian Council of Medical Research.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet **2018**; 392:951–70.
2. Ejazi SA, Bhattacharya P, Bakhteyar MA, et al. Noninvasive diagnosis of visceral leishmaniasis: development and evaluation of two urine-based immunoassays for detection of *Leishmania donovani* infection in India. PLoS Negl Trop Dis **2016**; 10:e0005035.
3. Patil RR, Muliyil JP, Nandy A, Addy A, Maji AK, Chatterjee P. Dynamics of the antibodies in cohorts of cured cases of visceral leishmaniasis: its implication on the validity of serological test, value in prognosis and in post therapeutic assessment. Hum Vaccin Immunother **2012**; 8:725–30.
4. Kühne V, Rezaei Z, Pitzinger P, Büscher P. Systematic review on antigens for serodiagnosis of visceral leishmaniasis, with a focus on East Africa. PLoS Negl Trop Dis **2019**; 13:e0007658.
5. Suresh HG, Pascoe N, Andrews B. The structure and function of deubiquitinases: lessons from budding yeast. Open Biol **2020**; 10:200279.
6. Damianou A, Burge RJ, Catta-Preta CMC, et al. Essential roles for deubiquitination in *Leishmania* life cycle progression. PLoS Pathog **2020**; 16:e1008455.
7. Azevedo CS, Guido BC, Pereira JL, et al. Revealing a novel otubain-like enzyme from *Leishmania infantum* with deubiquitinating activity toward K48-linked substrate. Front Chem **2017**; 5:13.
8. Didwania N, Ejazi SA, Chhajer R, et al. Evaluation of cysteine protease C of *Leishmania donovani* in comparison with glycoprotein 63 and elongation factor 1α for diagnosis of human visceral leishmaniasis and for posttreatment follow-up response. J Clin Microbiol **2020**; 58:e00213-20.
9. Ibarra-Meneses AV, Moreno J, Carrillo E. New strategies and biomarkers for the control of visceral leishmaniasis. Trends Parasitol **2020**; 36:29–38.
10. Gidwani K, Picado A, Ostyn B, et al. Persistence of *Leishmania donovani* antibodies in past visceral leishmaniasis cases in India. Clin Vaccine Immunol **2011**; 18:346–8.
11. Ejazi SA, Ali N. Developments in diagnosis and treatment of visceral leishmaniasis during the last decade and future prospects. Expert Rev Anti Infect Ther **2013**; 11:79–98.
12. Chappuis F, Sundar S, Hailu A, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol **2007**; 5:873–82.