Identification and Characterization of a Novel *Leishmania donovani* Antigen for Serodiagnosis of Visceral Leishmaniasis

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Abstract. Despite several drawbacks, rK39-based rapid immunochromatographic test is widely used for the diagnosis of visceral leishmaniasis (VL) in the Indian subcontinent. There is an urgent need to develop a better antigen. In this study we separated crude soluble antigens of *Leishmania donovani* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and hybridized with pool sera from pre- and post-treated VL patients, 6 months follow-up, endemic healthy (EHC), and nonendemic healthy controls (NEHC) by Western blotting. The sensitivity of enzyme-linked immunosorbent assay with identified protein was 95% (confidence interval [CI] = 89.6–98.01%), whereas the specificity for EHC, NEHC, and different disease groups were 96.3% (CI = 89.8–98.6%), 100% (CI = 95.8–100%), and 97.4% (CI = 91.02–99.3%), respectively. This specific antigen was subjected to two-dimensional gel electrophoresis and after tryptic digestion, antigen was characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Further analysis showed that it is a member of the heat shock protein family of 70 kDa, designated as BHUP1, and has great potential in the diagnosis of VL.

INTRODUCTION

Visceral leishmaniasis (VL), or kala-azar, is the most severe form of leishmaniasis. The gold standard for the diagnosis of VL is direct demonstration of parasites in tissue smears, which is invasive, risky, and requires considerable expertise. For several decades diagnostics have been developed to get a “field test” that is sensitive and easy to use in VL-endemic regions. Serologic tests like the direct agglutination test (DAT) was first to be used in endemic regions for the diagnosis of VL,^{1–3} however, a cumbersome procedure and prolonged incubation are its major drawbacks. The K39, a recombinant 39-amino acid antigen conserved in the kinesin region of *Leishmania chagasi* is now used as a rapid immunochromatographic test and has high sensitivity.^{4–6} However, in endemic regions, up to 32% healthy subjects react with the K39 strip test.^{7,8} Furthermore, it remains positive for several months to years, and thus cannot be used for diagnosing relapse.⁵ An antigen that detects active VL and differentiates it from asymptomatic infection is urgently needed. The objective of this study was to identify *Leishmania donovani* protein antigens with high specificity and sensitivity, and ideally turn negative as the patient recovers from the disease.

MATERIAL AND METHODS

Study site. The study was conducted at Kala-Azar Medical Research Center (KAMRC), Muzaffarpur, the field site of the Infectious Disease Research Laboratory (IDRL) of Banaras Hindu University, Varanasi, Uttar Pradesh. The study was approved by the Ethical Committee of the Institute of Medical Sciences, BHU, Varanasi. Informed written consent was taken from all participating volunteers.

Study population. Enzyme-linked immunosorbent assay (ELISA). Sera from 108 parasitologically confirmed VL patients > 2 years of age before start of treatment and from

47 individuals 6 months (Day 180) after end of successful treatment were collected. Control sera were collected from endemic healthy controls (EHC, $N = 82$) having no past history of kala-azar and nonendemic healthy controls (NEHC, $N = 87$). Seventy-seven sera samples were collected from different diseases (DD) consisting of malaria ($N = 28$), tuberculosis ($N = 25$), viral fever ($N = 15$), and liver abscess ($N = 9$).

Western blot. From the study subjects used in ELISA, 45 were selected randomly for Western blot. Sera from paired VL individuals were also collected at the end of successful treatment (Day 30). For each study group (Day 0, Day 30, Day 180, NEHC, EHC), nine panels were each made with a pool of five different sera.

Identification of antigen. A crude soluble antigen preparation of *L. donovani* parasite was first made, which was then run through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane and hybridized with the sera of different individuals.

Preparation of crude soluble antigen (CSA). The 1×10^8 parasites were harvested from stationary phase promastigote culture in cold $1 \times$ phosphate buffered saline (PBS) at pH 7.2 for CSA preparation. After washing and centrifugation, pellet was resuspended in $1 \times$ PBS and equal volume of complete protease inhibitor cocktail (Sigma) was added. Lysis of parasite cells was done by six alternate cycles of freezing (at -70°C), and thawing (at room temperature), followed by sonication. Supernatant was collected by centrifugation at 4,000 rpm for 10 min, and the protein was quantified by Bicinchoninic acid (BCA) kit (Thermo Scientific).

Antigen identification by Western blotting. The CSA (45 $\mu\text{g}/\text{well}$) of *L. donovani* was subjected on 12% SDS-PAGE, following the method of Laemmli.⁹ The CSA was immunoblotted according to Towbin and others,¹⁰ with a few modifications in Western blotting (Bio-Rad Mini-Protein II, Multiscreen), on PVDF membrane (0.45 μm pore size, Millipore) at 20 volts for 30 min. The membrane was treated with sera (1:100 in PBS) of different study groups, for 1 hour at room temperature. Alkaline phosphatase conjugated with goat anti-human IgG (1:1,000) was used as a secondary antibody. At the end, color was developed using BCIP-NBT (5-Bromo-4-Chloro-3-indolylphosphate + Nitro Blue Thiazole) as a

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substrate (Promega). The obtained bands were analyzed by Alpha Imager (Alpha Inno. Tech).

Partial purification of protein from SDS-PAGE gel. The specific protein band (70 kDa) from the SDS-PAGE gel was excised, crushed, and incubated overnight in an elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA, pH 7.5) at 37°C. The solution was centrifuged at 10,000 rpm, 10°C for 20 min, and the obtained supernatant was quantified for protein by the BCA method. We identified a novel *L. donovani* antigen (BHUP1) with the help of a very sensitive Western blot technique. We evaluated the sensitivity and specificity of this protein by enzyme-linked immunosorbent assay (ELISA).

Two-dimensional gel electrophoresis (2D-PAGE). Isoelectric focusing (IEF) was performed using immobilized pH gradient gel strips (IPG strips, Bio-Rad) with a pH range of 3–10. Five micrograms eluted protein was applied in 125 µL of rehydration buffer/IPG strip. The sample containing rehydration buffer was loaded overnight at room temperature by in gel reswelling under mineral oil to prevent oxidation of protein and drying of the gel strip. The loaded IPG strip was connected with the electrode of PROTIEN IEF cell (Bio-Rad, Gurgaon, India), followed by electric parameters 20 min, 100 V, and 50 µA; 30 min, 250 V, and 50 µA; 2 hours, 4,000 V, and 10,000 V for 3 hours. The IPG strip was then equilibrated in equilibrium buffer and run for a second dimension on resolving gel of SDS-PAGE. The gel was stained with a highly sensitive silver staining kit (Pierce Silver stain kit, Thermo Scientific) according to the manufacturer's instruction.

Mass spectrometry. The BHUP1 protein spot after silver staining was excised and subjected to protein sequencing analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry at The Center for Genomic Application (TCGA, New Delhi, India).

ELISA. The ELISA was performed as described elsewhere with some modifications.¹¹ Microtiter plates (Nunc, Denmark) were coated overnight at 4°C in carbonate buffer (pH 9.6) with eluted BHUP1 (70 kDa) protein (100 ng/well) of *L. donovani*

as a target antigen. To prevent nonspecific binding, the plate was blocked with 1% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. Sera (1:100 dilutions) of different study groups were added and incubated at 25°C for 1 hour. The serum antibody titers were measured with HRP conjugated goat anti-human IgG (1:16,000) secondary antibody, using tri-methylene benzidine (TMB, Promega) as a substrate. The reaction was stopped by adding 1 N H₂SO₄, and optical density (OD) was measured at 450 nm by an ELISA plate reader (Molecular Device, Spectromax 190). The cutoff value was determined by receiver operating characteristic (ROC) curve analysis for maximum specificity and sensitivity.

Statistical analysis. The ELISA data was analyzed through SPSS 16.0 software (SPSS, Inc., Chicago, IL). The comparative evaluation was done by a nonparametric, *t* test. The peak lists of the mass spectra were used for peptide mass fingerprint analysis with the Mascot software (Matrix Science; http://www.matrixscience.com/search_form_select.html) together with the National Center for Biotechnology Information (NCBI) sequence database. Protein was identified using the following parameters: database: eukaryote (eukaryotes); enzyme: trypsin; variable modification: oxidation (M), fixed modification: Carbamidomethyl (C); mass value: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±100 ppm, peptide charge state: +1, maximum missed cleavages: 1. The analyses of the post-source decay datasets were done by peptide mass fingerprint with Mascot.

RESULTS

Western blot. The pooled sera were tested for presence of antibodies against BHUP1 antigen. The soluble protein profile of the *L. donovani* promastigote obtained by SDS-PAGE showed a number of protein bands of different molecular weight. Several *L. donovani*-specific antigenic bands were recognized by the serum samples from different groups at different frequencies and intensities (Figure 1). The serum

FIGURE 1. Western blot of CSA (crude soluble antigen) with a panel of serum. Lane 1 = protein marker; D0 = pretreated; D30 = post-treated; D180 = 6 months follow-up; EHC = endemic healthy control; NEHC = nonendemic healthy control.

FIGURE 2. Eluted protein (EP) show a single protein after silver staining, Lane 1 = protein marker; Lane 2 = eluted protein (EP).

samples from patients with VL recognized 13 antigenic bands and the most frequently recognized bands (diagnostic for VL) are shown in (Figure 1). The Western blotting results indicate that all the pooled sera samples (9 of 9) from patients with acute VL showed a strong intensity of reaction with 70 kDa protein but it was recognized in only one (11.1%) of nine, 6-month follow-up pooled sera. None of the pooled sera panels from HEC and NEHC reacted with this protein.

The identified protein/(BHUP1) were eluted directly from SDS-PAGE gel (Figure 2), and similar antigenic response was observed with the eluted protein as that with CSA. Western blotting was repeated using the eluted antigen by hybridizing with a panel of same pooled sera, which included patients with active VL, Day 30, Day 180, EHC, NEHC, and patients with different diseases (malaria, tuberculosis, etc.) (Figure 3).

2D gel electrophoresis and MALDI-TOF characterization.

Two-dimensional gel electrophoresis from this eluted protein was performed to further characterize BHUP1. Two-dimensional gel electrophoresis profile revealed a single spot when stained with sensitive silver stain (Figure 4). The 2D spot was excised from the gel for MALDI-TOF characterization of the resolved protein and identified as heat shock protein of *L. donovani*

FIGURE 3. Western blotting result of 70 kDa (BHUP1) eluted protein Panel 1: 1 = nonendemic healthy controls (NEHC) (Pool), 2 = endemic healthy controls (EHC) (Pool), 3 = different diseases (DD) (Pool of 5 serum sample of malaria), 4 = 6-month follow-up serum, 5 = pool serum of after-treated patient, 6 = confirmed visceral leishmaniasis (VL) pool serum, 7 = blank; Panel 2: 8 = NEHC (Pool), 9 = EHC (Pool), 10 = DD (Pool of 5 serum samples of malaria, tuberculosis [TB], etc.), 11 = 6-month follow-up serum, 12 = pool serum of after-treated patient, 13 = confirmed VL pool serum. Circles contain bands of immunoreactive fraction BHUP1 with sera of pre- and post-treated patients.

FIGURE 4. Two-dimensional gel electrophoresis of BHUP1 eluted protein as stained with silver staining. Circle around the spot is our eluted protein.

of 70 kDa (Figure 5). Our protein sequence showed 100% homology with the sequence submitted in NCBI (accession ID X.52314) by Mac Farlane and others,¹² who identified an Hsp 70 complementary DNA (cDNA) in a cDNA library made from an African strain of *L. donovani*.

ELISA. The sensitivity of BHUP1 immunoreactive antigen was found to be 95% (confidence interval [CI] = 89.6–98.01%) for the detection of confirmed VL patients and specificity was 100% (CI = 95.8–100%), 96% (CI = 89.8–98.6%), and 97.4% (CI = 91.02–99.3%) in NEHC, EHC, and DD, respectively (Figure 6). The ELISA cutoff values for the immunoreactive BHUP1 fraction was defined on the basis of ROC (Figure 7) using sera obtained from NEHC ($N = 87$) as negative control and reactivity of all leishmaniasis patients as positive control. Furthermore, we compared BHUP1 with rK39 antigen for antibody response to 1 year follow-up cured patient's sera and found that our antigen was positive with 46% sera at

Protein sequence of identified immunoreactive fraction (BHUP1)

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1 MTFDGAIGID LGTTYSCVGV WQNERVDIIA NDQGNRTTPS YVAFDTSERL
51 IGDAAKNQVA MNPHTVFDA KRLIGRKFN D SVVQSDMKHW PFKVTTKGDD
101 KPMAVQYRG EEKTFTPPEI SSMVLLKMK E TAEAYLGKQV KKA VVTVPAY
151 FND SQRQATK DAGTIAGLEV LRIINEPTRA IAYGLDKGDD GKERNVLIFD
201 LGGGTDFVSL LTIDGGIFEV KATNGDTHLG GEDFDNRLVT FTEEFKRKN
251 KGKNLASSHR ALRGLRTACE RAKRTLSSAT QATIEIDALF ENVDFQATIT
301 RARFEELCGD LFRSTIQPVE RVLQDAKMDK RSVHDVVLVG GSTRI PKVQS
351 LVSDFFGGKE LNK S INPDEA VAYGA AVQAF I LTGGKSKQT EGLLLLDVTP
401 LTLGIETAGG VMTALIKRNT TIPTKKSQIF STYADNQPGV HIQVFEGERA
451 MTKDCHLLGT FDL SGIPPAP RGVPQIEVTF DLDANGILNV SAEKGTGKR
501 NQITITNDKG RLSKDEIERM VNDAMKYEAD DRAQRDRVEA KNGL ENYAYS
551 MKNTLGDSNV SGKLD DSDKA TLNKEIDVT L EWLSSNQEAT KEEYE HKQKE
601 LESVCNPIMT KMYQSMGGAG GMPGGM PDM SGMSGGAGFA GGASSGPKVE
651 EVD

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FIGURE 5. Protein sequence of identified immunoreactive BHUP1 protein. It is a 653 amino acid long having *Leishmania donovani* protein. NCBI Blast search result suggests that the protein is a heat-shock protein family.

FIGURE 6. Reactivity of sera determined by enzyme-linked immunosorbent assay (ELISA) with different study groups (visceral leishmaniasis [VL], nonendemic healthy controls [NEHC], endemic healthy controls [EHC], and different diseases [DD]). Full lines indicate average ELISA index (EI) for each assayed group. Dashed line represents cutoff value. Each dot represents an individual subject.

one year follow up, while rk39 showed 84% positivity in the same individuals.

DISCUSSION

A series of experiments were done to identify an immunoreactive BHUP1 antigen from *L. donovani*, which could be a potential marker in the diagnosis of VL. It turned out to be a heat shock protein that has never been used for the diagnosis of VL. Although rk39 is associated with high sensitivity (~99%), its specificity is a problem in the Indian subcontinent. A large proportion of healthy population, living in the endemic areas show positive with rk39.^{6,7} Most of the diseases like malaria, typhoid fever, tuberculosis, etc., whose signs and symptoms mimic VL and also show rk39 positive. Thus, there is an urgent need of a diagnostic that could specifically diagnose active VL. The BHUP1 immunoreactive antigen had a high specificity of 96% among healthy population living in the endemic region. Furthermore, more than half of the patients (54%) turned negative when tested with 1 year follow-up of cured VL patients as opposed to rk39 and DAT, which remained positive in 86% and 93% even after 1 year,¹³ respectively. Thus, *L. donovani*-specific Hsp 70 (BHUP1) protein also has potential to be used as a prognostic marker because most of the patients turned negative at 6 months. Because these experiments were done with antigen eluted from gel, though efforts were made to purify them by running in both 1- and 2-D PAGE, there is a probability of some impurities creeping in. Now that we have sequenced the amino acids, we will confirm the results with recombinant antigen in adequate sample size, before attempting to adopt it for a rapid immunochromatographic test. Because it remains positive in a significant proportion of patients at the end of treatment, it cannot be used for test of cure or relapses. Hsp 70 is a heat shock protein family and has been shown to act as a mitogen for the murine B cell.¹⁴ Wallace and others recognized a

FIGURE 7. Receiver operating characteristic (ROC) curve, cutoff determination for the enzyme-linked immunosorbent assay (ELISA) assay.

70 kDa heat shock protein (Hsp70) family as a major target of the humoral immune response during *L. donovani* infection.¹⁵ They observed that recombinant Hsp 70 of *L. donovani* was not recognized by sera of Chagas disease, cutaneous leishmaniasis, malaria, leprosy, or schistosomiasis. All of this indicates that immune response to Hsp70 is disease specific, and proves that this protein deserves particular attention in the field of anti-parasite immunity.

This finding is consistent with an earlier report that parasite Hsp is a prominent target of host immune response and anti-HSP antibody occurs in the patient.¹⁶⁻¹⁸ The BHUP1 show a high sensitivity and specificity with endemic-healthy control as compared with other pre-existing diagnostic markers. We conclude that we can use the recombinant 70 kDa antigen for the serological diagnosis of Indian VL.

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