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Analysis of Total Urine Proteins: Towards a Non-Invasive Approach for Diagnosis of Visceral Leishmaniasis

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Abstract

Diagnosis of Visceral Leishmaniasis (VL) is a major obstacle in the control of this disease, as demonstration of parasite by splenic or bone-marrow aspiration is almost impossible in the peripheral rural areas because of lack of facilities. In order to identify a non-invasive biomarker for VL, we employed two-dimensional electrophoresis (2D-PAGE) coupled with mass spectrometry through a comparison of urinary proteome. Though, we did not find the biomarker protein for VL, our findings provide some basic insights for future development of non invasive diagnostic tool.

Keywords: Visceral leishmaniasis; 2D-PAGE; Urinary proteins; MALDI-TOF

Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a chronic infectious disease caused by obligate intracelluar parasites of the genus Leishmania and accounts for enormous morbidity and mortility amongs poor people living in rural areas. The estimated annual incidence of VL is 200 to 400 thousands and 90% of these occur in India, Bangladesh, Nepal, Sudan, Ethiopia and Brazil [1]. If left untreated, VL is fatal and its clinical presentation is defined by non-characteristic symptoms (chronic fever with hepato-splenomegaly). As VL mainly affects remote rural communities, its diagnosis requires point-of-care (POC) tools that are easy to use, cheap and available in the peripheral health facilities. Since, anti-leishmanial drugs are toxic and expensive, an accurate biomarker for diagnosis and prognosis of VL is urgently needed.

Demonstration of parasites in splenic or bone marrow biopsies, and antibody detection by rK39 strip test are the tools commonly used for diagnosis of visceral leishmaniasis (VL) [2]. Bone marrow and splenic aspiration are painful and risky procedures, and serology with rK39 rapid immunochromatographic test can be positive in 14 - 32% healthy individuals living in the endemic areas [3]. Antibody based tests have lower sensitivity in HIV co-infected patients, and cannot be used in relapses or for assessment of cure as they remain positive for long periods after cure. An antigen based diagnostic test could overcome these pitfalls, however, the high levels of circulating antibodies and immune complexes in VL patients makes it difficult to develop such tests using serum. For both clinical and epidemiologic purposes, non-invasive urine sampling may be more convenient than blood. Therefore, we took a proteomics approach for the identification of biomarkers for VL by using urine samples and compared urine proteome maps of VL patients before and after treatment. The work presented here provides a framework of 2D gel and mass spectrometry based approaches for detection of antigen excreted in urine of active VL. The primary goal of our proteomic effort was to identify the proteins directly or indirectly involved in VL.

Materials and Methods

Urine samples were collected from Kala-azar Medical Research Centre (KAMRC) Muzaffarpur, Bihar, field site of Banaras Hindu University, Varanasi, India. The Ethics committees of the centre and Banaras Hindu University approved the protocol. Written informed consent was obtained from all of the subjects prior to enrollment in the study. Urine of five patients with VL before and after completion of treatment and at six month follow up were used for proteomic analysis. Urine from five healthy controls (from Bihar, India) and those suffering from other febrile illnesses (n=5) were also tested. Participating subjects endemic healthy were asked to provide freshly voided urine in 15-mL test tubes. These urine samples were centrifuged at 2000 × *g* for 10 min for removal of cell debris and stored at -80°C until use. Total urine proteins were isolated by ITSI column (ITSI Bioscience ToPI-U , USA), and its concentration was estimated using the BCA method (Thermo Scientific, USA) [4]. Isoelectric focusing (IEF) and SDS–PAGE were performed using the mini-protean 2DE system (Biorad, USA). In first dimension (IEF), samples (25 µg total urine proteins premixed with rehydration buffer) were run on 7 cm long (Bio Rad) immobiline DryStrip, (linear pH 3-10) in Ettan IPGphor II IEF System (Bio Rad) at 20°C, using stepwise mode to reach 10000 V/hours. The maximum current setting was 50 mA per strip and IEF run was carried out using the following conditions: 250 V gradient (20 min), 4000 V gradient (2:30 hr), and 4000V rapid (1000 V/hr). Following IEF, strips were sequentially incubated for 15 min each in equilibrium buffer-I (BioRad) and equilibrium buffer- II (BioRad). The IPG strip were then transferred onto 10% SDS-PAGE slab gel (8 X 9.5 cm) and the second dimensional separation were performed in Mini-Vertical Electrophoresis Unit (Bio Rad) with the current 20 mA/gel for 2.5 h. Separated protein spots were then visualized by silver stain. Digital images of the protein maps were recorded using the Alpha Innotech gel documentation system and comparative analysis of digitized proteome maps was performed using PD – Quest Image analysis software (BioRad). Differentially expressed protein was identified and subjected to MALDI-TOF followed NCBI-BLAST and MASCOT search.

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Results and Discussion

Resolution of urine proteins on one-dimensional SDS-PAGE was poor and variable (Figure 1). Inspection of the 2D profiles of urine proteins (Figure 2) suggested that the degree of apparent similarity in protein profiles can be correlated to the proteins in question. Because the urinary proteome is variable, even from the same individual at different time points, we also looked and analyzed the individual urinary proteome. Thus, we compared the overall features of the urinary proteins between single and pooled specimens using the master maps generated by the PD-quest software.

Analysis of proteome map over a pH of 3-10 aligned a total of 89 proteins by PD-quest software and only one spot (SSP 9101) was found to be up regulated in urine of all five VL patients compared to EHC (Figure 2). Further, this spot was positive in 40% and 20% after post-treatment and 6 months followed up urine samples, respectively. However, presence of this spot (40%) in patients with other diseases (malaria and tuberculosis patients) preclude as a diagnostic marker. This spot was identified as myosin-reactive immunoglobulin light chain by MALDI-TOF (Figure 3).

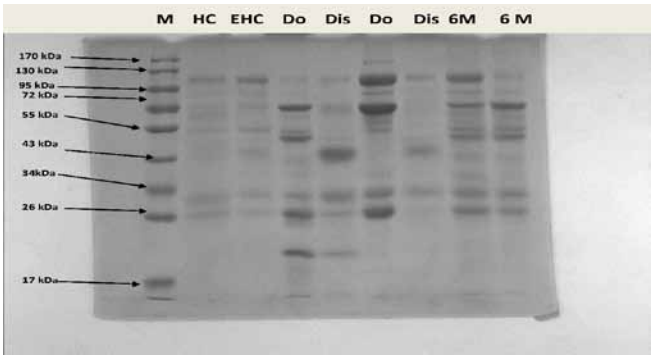

Figure 1: Urinary proteins separation on one-dimensional SDS gel: Urinary protein (25 µg/lane) from single sample and pooled samples were applied on a 12% acrylamide gel. Gel was stained by colloidal Coomassie. M = molecular marker; HC= healthy controls from non-endemic regions; EHC = endemic healthy controls; D0 = active VL patients (day-0); Dis = Patients after 30 days treatment; 6M = cured VL after 6 month follow-up.

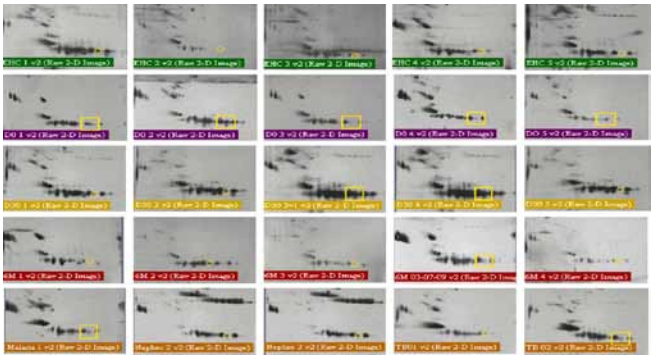

Figure 2: 2DE map of total urine proteome: Proteins were separated by IEF in the neutral range (pH 3–10) followed by gradient 10% SDS–PAGE, and the resulting 2DE protein arrays were detected by staining with silver stain. Solid circles indicate absence of SSP 9101 while presence of this protein spot is indicated by square. EHC = endemic healthy controls; D0 = active VL patients (day-0); Dis = Patients after 30 days treatment; 6M = cured VL after 6 month follow-up; TB = tuberculosis patients.

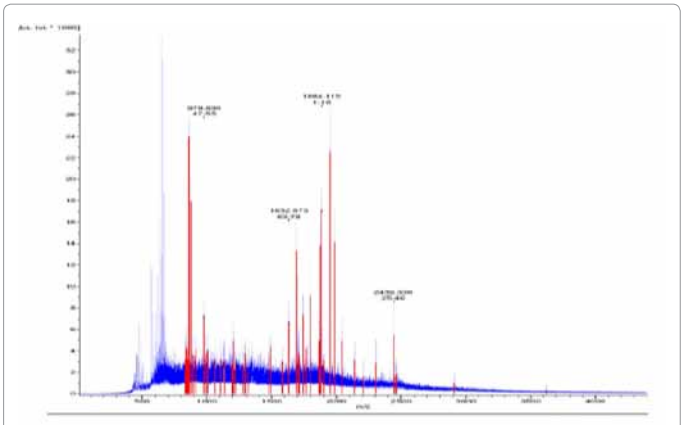

Figure 3: MALDI-TOF spectrum of peptide from VL patients: The protein spot in the silver-stained gel that had been assigned to antigen spot SSP-9101 in 2 D gel were excised, distained and incubated with trypsin. The resulting fragment was analyzed by MALDI-TOF/MS. Upon processing via MASCOT, the antigen was identified as myosin-reactive immunoglobulin light chain.

To the best of our knowledge this is the first study for identification of biomarkers in urine of VL patients by comparing total urine protein maps. The detection of antigen in the urine is increasingly being used for diagnosis of parasitic infections. A urinary antigen has recently been demonstrated in visceral leishmaniasis (VL), using a latex agglutination test, the only commercially available urine test (KAtex) for detection of VL with varying results [5,6]. Studies in the Indian subcontinent and different African countries have demonstrated a low sensitivity of KAtex (55.6 % – 67%) [7,8]. This is a major limitation of KAtex as a reliable tool. Though, still a workhorse in proteomics, 2-D gels are limited to the identification of relatively abundant soluble proteins. A new method for identifying large number of proteins from complex samples need to be developed. Though, we did not find any clear biomarker protein which could differentiate VL patients to EHC or different disease patients, but advances in active spectrometry and proteomics based approaches hold great promise as the basis for new diagnostic tests and as a mean to better understand disease pathogenesis.

Acknowledgements

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