Diagnostic and Prognostic Applicability of an Ubiquitin like 13kDa *Leishmania donovani* Excretory-Secretory Protein for Visceral Leishmaniasis

Jalaj Kumar Gour[1], Haushila Prasad Pandey[2], Dhiraj Kishore[3], Sangram Singh[*4], Vinod Kumar[*5]

[1]Department of Biochemistry, Faculty of Science, University of Allahabad, Prayagraj, India.
[2]Department of Biochemistry, Nepalgunj Medical College (NGMC), Chisapani Campus, Nepal.
[3]Department of Medicine, Institute of Medical Science, Banaras Hindu University, Varanasi, India.
[4] Department of Biochemistry, Faculty of Science, Dr. RML Avadh University, Faizabad, UP, India.
sangram_rml@yahoo.co.in
[5]Department of Molecular Biology, Rajendra Memorial Research Institute of Medical Sciences, Agamkuan, Patna, Bihar, India. vinod44biogene@gmail.com

Abstract: **Prognostic tool or a test of cure to monitor drugs response is not available for leishmaniasis till date. Individuals suffering with visceral leishmaniasis (VL) show seropositivity to all currently used diagnostic antigens even after years of successful cure. In this study, we explored a 13kDa excretory-secretory component of *L. donovani*, which showed seronegativity with serum obtained from patients on completion of treatment. *Leishmania donovani* promastigotes were grown in Dulbecco's modified eagle media. The leishmanial excretory-secretory antigens (LESAs) were prepared and recovered from parasites free culture supernatant. The diagnostic and prognostic applicability of 13kDa protein was evaluated by enzyme linked immunosorbent assay.**

Result indicates that patient's sera bears seropositivity for 13kDa protein before treatment and found to be antibodies negative after completion of treatment. The sera samples of various controls did not show seropositivity with this protein. The ELISA sensitivity of this protein was observed to be 100% at absorbance cut-offs 0.180 and 0.360. The specificity at absorbance cut-off 0.180 in non-endemic, endemic and disease control groups was observed to be 98%, 92% and 94%, respectively. The homology modeling predicted its homology with ubiquitin like protein. The identified protein was found to be highly sensitive and specific for serum antibodies present in VL patients. The findings also indicated its prognostic potential, which can also be exploited to monitor drug responses.

Keywords: **Leishmaniasis, ubiquitin, diagnosis, prognosis.**

I. INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by the Leishmania parasite, which affects about 98 countries. (Alvar et al., 2012). The infection of *Leishmania* in humans manifest mainly in three clinical forms namely the cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and the fatal form visceral leishmaniasis (VL), that represent considerable degrees of difference in their immunopathologies. According to a report approximately 350 million people worldwide are on the verge of latent infection of all types of leishmaniasis, and about 2 million fresh cases are being reported each year (Bhargava and Singh., 2012). Approximately 500,000 new cases of VL or KA (kala-azar), which is mainly caused by *L. infantum* and *L. donovani* are being reported annually. Among three major forms of leishmaniasis, VL is the most devastating one if left untreated (Desjeux, 2004). It is reported that about 200 million individuals are exposed for VL infection in disease endemic regions of Indian subcontinent (Stauch et al., 2011). Due to unavailability of a vaccine and very limited drug options there is an urgent requirement of accurate diagnostic and prognostic tools to curb diseases complications and burden.

An early and accurate diagnosis not only helps in effective management of disease but also reduces the chances of disease spread and parasitic resistance (Tiwari et al., 2018 ; Singh et al., 2012) The demonstration of parasite either in the bone marrow

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* Corresponding Author

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or splenic aspirate of affected individuals is the diagnostic gold standard for visceral leishmaniasis (VL) which is invasive and quite risky (Gao et al., 2015). Various serum based tests such as rk39 strip test, direct agglutination test (DAT), and enzyme linked immunosorbent assay (ELISA) are being widely used for detection of antileishmanial antibodies but their sensitivity and specificity is highly compromised in endemic regions (El-Moamly et al., 2012; Boelaert et al., 2008). Moreover, due to presence of persistent leishmanial antibodies in patients serum even after successful treatment, their prognostic capabilities are also compromised. In addition, they can not be used to confirm the cases linked to re-infection or reversion. In recent years various antigens detection methods have been developed such as polymerase chain reactions (PCR) (Srivastava et al., 2011), direct detection of urinary antigens either by immunoblotting (Kumar et al., 2011) or agglutination based (Katex) methods (Ahsan et al., 2010). Although these methods have shown excellent sensitivity but require expertise, time and sophisticated laboratory facilities, and are also unfriendly for large scale population diagnostic requirements.

During the various stages of life cycle, *Leishmania* excretes or secretes several factors or products in its surrounding environment within the hosts however, their identification and characterization is not fully done (Chenik et al., 2006; Rajasekariah et al., 2007). The most remarkable effort for identification of antigenic excretory secretory (ES) protein has been done by Chenik and colleagues. They have screened thirty three (33) ES proteins by means of cDNA and immune sera of rabbit raised in response to ES proteins. Out of 33 ES proteins, 9 have been identified as ES protein in *Leishmania* or other related species. Among all, 11 proteins are considered as known proteins but they are not identified as secreted and rest thirteen (13 ES proteins) were characterized as novel proteins (Chenik et al., 2006). In various parasitic diseases such as Chagas disease, filariasis, *Angiostrongylus cantonensis* etc. diagnostic capability of ES proteins has been well documented though it is not yet evaluated in any form of leishmaniasis (Berrizbeitia et al., 2006; Madathiparambil et al., 2009). Here, we present the identification and characterization of 13kDa *Leishmania donovani* ES protein, and its diagnostic and prognostic prospects in visceral leishmaniasis.

II. MATERIAL AND METHODS

A. Study Design

Briefly, Excretory-secretory (ES) proteins of *L. donovani* were prepared and then were separated on SDS-PAGE. Resolved proteins on gel were transferred to PVDF membrane then incubated with sera of healthy individuals, those treated for leishmaniasis, the individuals from endemic, non-endemic and disease (tuberculosis-10, malaria-30, enteric fever-5, splenomegaly-5) controls. The diagnostic applicability of 13kDa ES protein was evaluated by ELISA. The LC-MS/MS analysis,

phylogenetic and protein modeling were performed to characterize this protein (Figure 1).
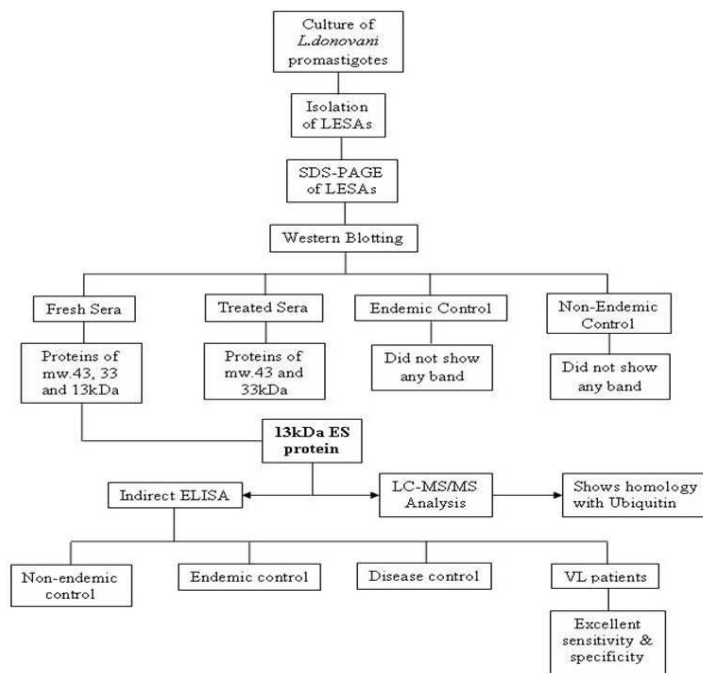

Figure 1: Study design diagram.

B. Culture of Leishmania parasite

Leishmania donovani (MHOM/IN/80/Dd8) promastigotes were grown in Dulbecco's Modified Eagle Media (pH7.2) (DMEM, Invitrogen, USA) composed of 10% heat-inactivated fetal bovine serum (Gibco), 2mM L-glutamine, sodium bicarbonate, penicillin (100U/ml), streptomycin (100µg/ml), gentamicin (20µg/ml) (Sigma Chemicals, USA) at 26°C in a BOD incubator. Parasites in the form of motile promastigote were taken throughout the study.

C. Isolation of Leishmanial excretory-secretory antigens (LESAs) from L. donovani promastigotes

The leishmanial excretory-secretory antigens (LESAs) were prepared by taking stationary phase promastigotes having a parasite count of nearly about 2-3 x 10^8 promastigotes per ml. In brief, *Leishmania donovani* promastigotes were pelleted by centrifugation at 2000 rpm for 20 min at 4^0C and further for the removal of FBS it was washed 4-5 times repeatedly by centrifuging each time. For obtaining LESAs, about $3x10^8$ promastigotes per ml were maintained and incubated for 24 hours in serum free media (pH 7.2, 26^0C). Viability and integrity of parasites prior to LESA isolation were tested by dye exclusion test using trypan blue and by observing motile promastigotes under phase contrast microscope. The incubated parasites were removed by centrifuging at 2000 rpm and supernatant was passing through 0.22µm filter unit (Millipore, USA).

Supernatant containing LESAs was concentrated about ~80-100 times by using 3kDa-cutoff ultra-filtration unit (Amicon Ultra, Millipore, USA). Protein concentration was estimated following Lowry et al. (1951) using bovine serum albumine to prepare calibration curve. LESA protein sample was then stored at −80℃ until execution of further experiments. (Lowry et al., 1951).

D. SDS-PAGE and Immunoblotting

The isolated LESA proteins were separated on 12% SDS-PAGE (Laemmli, 1970) and proteins were stained with silver staining method (Schagger H, 2006)**.** Molecular weight marker protein mixture (Fermentas SM0671; 10-170kDa) was run in parallel lane to estimate the molecular weight of unknown proteins in sample lanes. Gel was then observed under gel documentation system (Alpha Innotech Corporation, USA). For immunoblotting, LESA proteins from unstained proteins were transferred onto PVDF membranes according to the standard protocol. After washing repeatedly for three times with blocking buffer having PBS/Tween-20, the membranes were treated with sera (1:100 dilutions) obtained from patients (before and after completion of treatment, endemic control and non-endemic control) in blocking buffer for 2hrs at room temperature (RT). Thereafter, membranes were washed thrice with PBS/Tween-20to remove unbound antibodies, and incubated with a 1:3000 dilution of anti-human immunoglobulin G conjugated with alkaline phosphatase (Sigma, Aldrich, USA) prepared in blocking buffer followed by three additional washes. BCIP/NBT was used for developing color and the reaction was stopped by adding triple distilled water. After development, membranes were photographed and analyzed by gel documentation system.

E. Recovery of 13kDa protein for ELISA

Recovery of LESAs was done by reverse staining method following Castellanos-Serra et al., (1997). Initially, the crude proteins were first separated on 12% SDS PAGE, and after rinsing in MilliQ water the gel was treated with 0.2M imidazole solution containing 0.1% SDS for 10 minutes. Further, the gel containing proteins was immersed in 0.2M zinc sulphate solution for 5 minutes for the development of colorless bands on a white background. At last, water was added to stop the reaction. The gel strips were rinsed with 0.02 M PBS (pH 7.2) for 10 minutes, which was followed by rinsing of gel in 100mM EDTA solution to complex zinc ions for recovery of proteins. Further, the gel was treated with 0.1% Triton X-100 to remove excess SDS and to renature the proteins. The protein was eluted by crushing and shaking the gel thoroughly in least amount of PBS and finally filtered through 0.22µm filter unit to remove the impurities. Protein was assayed and stored at -80°C until further use.

F. Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA was performed using the standard procedure described by Voller (1978). Briefly, 96 well flat bottom plates (Nunc, Germany) were coated with purified ES protein at concentration of 10µg/ml in the coating buffer (carbonate-bicarbonate buffer, pH 9.6) and plates were incubated at 4ºC overnight. Thereafter, the wells were washed thrice with PBS/Tween 20 (0.1%) and blocked with 2% non-fat dry milk in PBS-Tween 20 for 2hrs at RT. After incubation, plates were again washed as previously and incubated with serum samples of VL and cured patients, endemic, non-endemic and disease controls. After 2 hrs of incubation at RT, plates were washed for three times and subsequently incubated with HRP-conjugated rabbit anti human immunoglobulin (IgG-HRP) (1:5000 dilution) for 2 hrs at RT and washed with wash buffer three times after incubation. The color was developed by adding orthophenylene diamine dihydrochloriode (OPD) as substrate and reaction was stopped by 1N H_2SO_4. The plates were read at 450nm in an ELISA plate reader (BioRad, USA). ELISA was performed on pre-stored fifty (50) serum samples of each category. The sensitivity and specificity of the 13kDa protein was calculated at 0.180 and 0.360 absorbance cut-offs using following formulas:

Sensitivity = true positives/true positives + false negatives x 100and

Specificity = true negatives/true negatives + false positives x 100.

G. LC-MS/MS analysis and prediction of antigenicity

LESAs were excised from gel and submitted to National Institute for Plant Genome Research (NIPGR), New Delhi, India for LC-MS/MS analysis for protein identification. The antigenicity was calculated following Kolaskar and Tongaonkar (1990).

H. Phylogenetic analysis and protein modeling

Sequence of *L. donovoni* protein was identified by LC-MS/MS analysis and further phylogenetic analysis was carried out by Clustal-W and Espript tools. Obtained protein sequence was submitted to BLAST search for the prediction of homology with sequences present in NCBI database. From there, homologous sequences were selected and aligned by Clustal-W algorithm. Sequence alignment with specific color-codes was generated using Jal view. Sequence of *L. donovani* protein was submitted in to I-Tasser sever, which uses fold recognition method for providing the best modeled structure.

III. RESULTS

A. Antigenicity of ES proteins of Leishmania donovani parasites

A total of 17 proteins (11-80kDa) were observed in SDS-PAGE gel after silver staining of which the low molecular weight proteins were found to be highly expressed as compared to high molecular weight proteins (Figure 2 lane A). The immunoblotting of proteins with patient's sera confirmed their antigenicity (Figure 2 lane B). Three proteins having the molecular weights 13kDa, 33kDa and 43kDa were seropositive

(Figure 2 Lane C) on immunoblotting with sera obtained prior to treatment however, the 13kDa protein seropositivity was absent on immunoblotting with sera obtained from individual who completed the treatment (Figure 2 Lane D). Blotting with sera obtained from endemic, non-endemic and disease controls did not show any seroreactivity with this protein, which confirmed that 13kDa ES protein is disease specific.

B.   ELISA estimated sensitivity and specificity of 13kDa ES protein suggested its excellent diagnostic and prognostic potential

The diagnostic and prognostic potential of 13kDa protein was evaluated by its sensitivity and specificity in ELISA test at two absorbance cutoffs i.e. 0.180 and 0.360. The sensitivity of ELISA was observed 100% at both absorbance cut-offs in VL patients. The specificity at OD cut-off 0.180 in non-endemic, endemic and disease controls was found to be 98%, 92% and 94%, respectively. However, at higher absorbance cut-off (0.360), the specificity was 100% in non-endemic control samples followed by endemic and disease controls (96%) (Table 1).
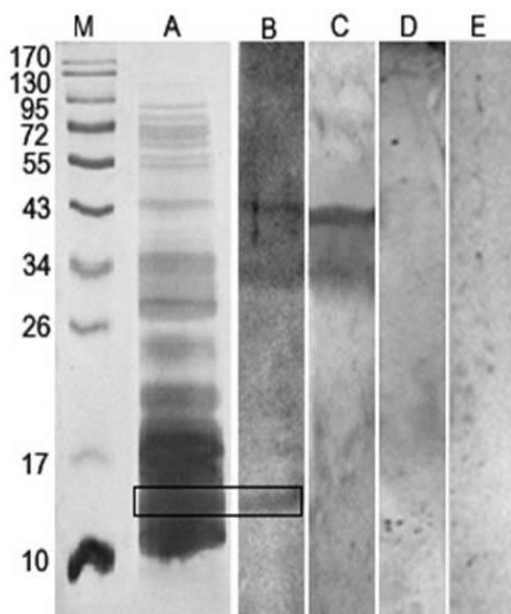

Figure 2: Silver-stained gel (12% SDS-PAGE: 40 μg/well) of leishmanial excretory-secretory (ES) proteins; Lane A: crude lysate of ES proteins; Lane B: immunoblot of ES protein with VL patient serum;, Lane C: immunoblot of ES protein of treated individuals serum; Lane D: immunoblot of ES protein with serum of endemic control; Lane E: immunoblot of ES protein with serum of non- endemic control;   M: molecular weight marker.

Table1: ELISA performed with 13kDa protein treated with serum samples of visceral leishmaniasis, healthy endemic and non-endemic controls and disease control i.e.   patients with confirmed other diseases.

Groups	n	Absorb-ance range	mean absor-bance±SD	Specificity (%) at cut-off absorbance		Sensitivity (%) at cut-off absorbance	
				0.180	0.360	0.180	0.320
Non-endemic control	50	0.018-0.215	0.061± 0.048	98	100	-	-
Endemic control	50	0.032-1.921	0.130± 0.298	92	96	-	-
Disease control	50	0.022-1.980	0.126± 0.325	94	96	-	-
VL patients	50	0.525-2.716	1.751± 0.518	-	-	100	100
VL cured	50	0.032-1.7580	0.357± 0.3916	100	100	-	-

C.   Sequence Homology and antigenicity prediction

The sequence alignment of complete ubiquitin sequences (128aa) revealed that from position 1 to 76 this protein is highly conserved in *Leishmania* species and human. However, from position 77 to 128, it was conserved within *Leishmania* species and was found significantly distinct from human (Figure 3A). The 3D structure of identified leishmanial protein was not found in protein data bank (PDB), therefore ab-initio was used for protein modeling. The online available server (I-tasser) was used for prediction of 3D structure of protein (Figure 3B). We predicted 5 models on the basis of C-scores value and also used the structure assembly simulations parameter along with trading template alignments tool for C-score calculations. The predicted model has 3 helix and 2 beta-sheets and loops, also.   The Kolaskar and Tongaonkar antigenicity prediction method indicated that the amino acid sequences from 77 to 128 are mainly responsible for antibody production (Figure 4).

IV.   DISCUSSION AND CONCLUSION

The amastigotes demonstration in smear of bone marrow and spleen is still a gold standard method for diagnosis of visceral leishmaniasis. During recent years various serological tests like rk39 strip test and DAT have been developed and also being used as a tool to start a therapeutic regimen mainly because of their excellent sensitivities (Sundar et al., 2006 ; Singh et al., 2006). However, due to persistent antibodies for these antigens even after successful treatment are the main drawbacks of these tests because they can not be used as test cure. Report says that rk39 antigen detect antileishmanial antibodies even after 12 years of successful treatment (Gidwani et al., 2011 ; Silva et al.,

2006) and DAT detect up-to 7 years (Hailu., 1990). Moreover, the cases of sub-clinical infections (with few symptoms or no symptoms with positive serology) in which tissue smears are often negative for parasites, are very common in endemic areas also limits their diagnostic usefulness (Marty., 1994).
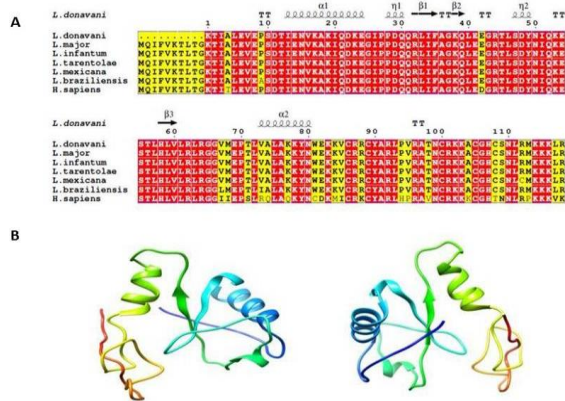

Figure 3:**A.** The alignment of amino acid sequence of 13kDa protein of *L. donovani, L. major, L. infantum, L. tarentolae, L. Mexicana, L. braziliensis and H. sapiens*. KEGG was used for retrieving the sequence of amino acid and the Clustal-W and ESPript 2.2 software was used for alignments. The secondary structure of protein (α helices and β sheets) is mentioned in above sequences. The highlighted shaded boxes are considered as invariant residues. **B.** Predicted 3D structure of 13kDa ES protein of *L. donovani.*

Antigenic plot for sequence

Antigenic determinants of the sequence

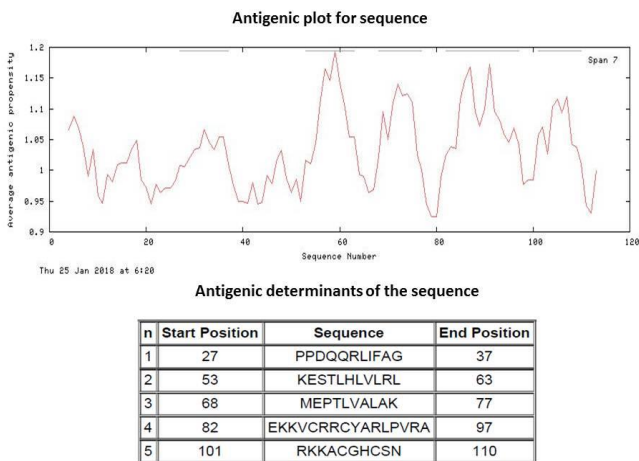| n	Start Position	Sequence	End Position
1	27	PPDQQRLIFAG	37
2	53	KESTLHLVLRL	63
3	68	MEPTLVALAK	77
4	82	EKKVCRRCYARLPVRA	97
5	101	RKKACGHCSN	110

Figure 4: Antigenicity plot of 13kDa protein sequence (110 amino acids) showed five different antigenic determinant regions ranging from 27-37, 53-63, 68-77, 82-97 due and 101-110 residues.

In addition, their specificities are also compromised as they show cross reactivity with other disease like tuberculosis, malaria, amoebiasis, sleeping sickness etc. (Van Etten, et al.,

1994; Porrozzi et al., 2007; Romero et al., 2009). Hence a diagnostic and prognostic test that excludes all these limitations is necessarily required.

In this study, the identified 13kDa protein showed excellent diagnostic accuracy in relation to both, specificity and sensitivity. The absence of cross reactivity with serum samples for other diseases confirms that the leishmanial ES protein responsible for antigenicity are highly conserved in *Leishmania* species as revealed by antigenicity prediction. Further, it showed seroreactivity with sera of visceral leishmaniasis patients and did not illustrate any seroreactivity with sera of treated patients that strongly put forward its prognostic utility. LC-MS/MS analysis of ES protein shows homology with ubiquitin, which is a low molecular weight protein (8.5kDa) conserved in all eukaryotes. Ubiquitin plays a key role in many biological processes and regulates cell cycle replication, stress response, DNA repair, organelles biogenesis and signal transduction. (Feng et al., 2007; Gannavaram et al., 2011).

Excretory-secretory molecules of organisms are interacts with host immune cells. Parasite also releases molecules that help in evasion from host immune response, penetration into tissue, attainment of nutrients and oxidative stress response. The ES product of parasite may be used as potential diagnostic tool due to their appearance at parasite-host interaction and their proven role in immune system. It may also be beneficial in understanding the parasite host interaction.

To the best of my knowledge, this is the first study to demonstrate leishmanial ES protein which is immunogenic and may be used for development of diagnostic and prognostic tools.

Recently, many leishmanial antigens have been identified for their use in diagnostic and prognostic applicability to improve serological diagnosis of VL. Among these, some antigens such as histones, gp63, KMP11 or LACK, ribosomal proteins, HSP70, HSP83 are characterized well and have been used for protocols for vaccination programme or as diagnostic tools. , (Guptaet al., 2007; Kubarand Fragaki2005; Rodríguez-Cortés et al., 2007). However, a test on the basis of these antigens is still far from reality. Primary results of our study seem to be very convincing for development of new diagnostic tool but require further evaluation on large sample size. In conclusion, the identified leishmanial ES protein may be used to explore a precise diagnostic assay and probably an efficient vaccine to prevent transmission and spreading of VL from every corner of world.

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