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RAPID COMMUNICATION

Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes

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Visceral leishmaniasis (VL) caused by *Leishmania donovani* is a major parasitic disease prevalent in endemic regions of Bihar in India. In the absence of good chemotherapeutic options, there is a need to develop an effective vaccine against VL which should be dependent on the generation of a T helper type 1 (Th1) immune response. We have shown that soluble proteins from promastigote of a new clinical isolate of *L. donovani* (2001) ranging from 68 to 97.4 kDa (F2 fraction), induce Th1 responses in the peripheral blood mononuclear cells of cured *Leishmania* patients and hamsters and also showed significant prophylactic potential. To understand the nature of F2 proteins, it was further characterized using 2-DE, MALDI-TOF and MALDI-TOF/TOF-MS. In all, 63 spots were cut from a CBB stained gel for analysis and data was retrieved for 52 spots. A total of 33 proteins were identified including six hypothetical/unknown proteins. Major immunostimulatory proteins were identified as elongation factor-2, p45, heat shock protein (HSP)70, HSP83, aldolase, enolase, triosephosphate isomerase, protein disulfideisomerase and calreticulin. This study substantiates the usefulness of proteomics in characterizing a complex protein fraction (F2) map of soluble *L. donovani* promastigote antigen identified as Th1 stimulatory for its potential as vaccine targets against VL.

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Visceral leishmaniasis (VL or Kala-azar) is the most devastating type among the spectrum of diseases caused by parasites of the *Leishmania* family. It is caused by the invasion of the reticuloendothelial system (spleen, liver and bone marrow) by the hemoflagellate protozoan parasite *Leishmania donovani*. The disease is restricted to areas which are heavily infected by the sandfly (*Phlebotomus spp.*), the vector of this disease which is widely distributed in the Indian subcontinent and south-west Asia [1, 2]. In India, Bihar, Assam, West Bengal and Eastern Uttar Pradesh are endemic for VL. The available anti-leishmanial drugs are toxic, have serious side-effects and are associated with numerous relapses and there is an increasing incidence of drug resistance [1]. In the absence of suitable anti-leishmanial drugs, an alternative choice for the control of this disease is immunoprophylaxis. The search for parasite antigens able to induce immune re-

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Abbreviations: HSP, heat shock protein; **SLD,** soluble *Leishmania donovani* promastigote antigen; **Th1**, T helper type 1; **VL**, visceral leishmaniasis

sponse has been predominantly associated with the identification of proteins that may be used for vaccine development. Most studies aimed at identifying antigens from *Leishmania* spp. have searched for molecules with the ability to stimulate IL-2, IFN-γ and IL-12 [3–7]. Our previous studies have shown that soluble non-membranous *Leishmania donovani* proteins (SLD) are potent activators of T helper type 1 (Th1) immune responses [8]. Using ammonium sulphate fractionation, preparative SDS-PAGE and electro elution techniques, we have shown that the 68–97.4 kDa fraction (termed F2) elicited strong T cell responses in persons who had recovered from VL, as well as in cured hamsters. In addition, the F2 fraction in combination with the *Bacille Calnette Guerin* (BCG) vaccine showed considerably good prophylactic activity against *L. donovani* challenge [8]. These observations have led to the necessity to dissect the F2 fraction to identify the specific constituents or proteins which are immunostimulatory.

Proteomics is a useful tool for obtaining a more complete understanding of the biology of *Leishmania*. This study was planned to apply 2-DE based peptide mass-mapping for the characterization of the F2 proteome. This approach has been found to be useful for analyzing the proteomes of some parasitic organisms, *e.g. Trypanosoma gondii* [9], *Fasciola hepatica* [10], *T. cruzi* [11], *Leishmania* sp including its development, evolution and pathogenicity [12–15]. 2-DE combined with the immunoblot assay also enabled detection of many antigenic profiles such as of *T. gondii* [16, 17], *Schistosoma japonicum* [18], *Ascaris suum* [19], *Neospora caninum* [20, 21], *Candida albicance* [22]. Some proteomic studies have also been done to identify the potent T cells activator proteins of *Mycobacterium tuberculosis* [23, 24].

The *L. donovani* (strain 2001) used in this study was cultured *in vitro* and *in vivo* as described previously [8, 25]. SLD was prepared as per method described by Scott *et al.* [26] and modified by Choudhury *et al.* [27]. Briefly, metacyclic promastigotes (10^9) were harvested from culture, washed four times in cold PBS, resuspended in PBS containing protease inhibitors cocktail (Sigma, USA) and subjected to ultrasonication and centrifugation at $40\,000 \times g$ for 30 min. The protein content of the solutions was estimated [28].

SLD was precipitated in TCA to a final concentration of 10% w/v and washed three times with acetone to eliminate contaminants like nucleic acid and salts (by employing PBS or Tris-HCl buffer). The dry pellet was solubilized in rehydration buffer (7 M urea, 4% w/v CHAPS, 100 mM DTT, 0.5% v/v biolyte (3–10) and 40 mM Tris) and vortexed and centrifuged to remove insoluble material. Immobline Dry Strips, p*I* 3–10, 17 cm (BioRad, USA) were loaded with 1.5 mg of SLD and allowed to rehydrate for 18–22 h. IEF was performed at 20°C using the Protean IEF cell (BioRad) according to the manufacturer's instructions. After IEF, the strip was equilibrated in solution A (0.375 M Tris, pH 8.8 containing 6 M urea, 2% SDS, 20% glycerol, 2% w/v DTT) and B (solution A without DTT, but with 2.5% w/v iodoacetamide) for 20 min at room temperature and inserted on to a 12% 2-D SDS-PAGE gel (20 × 22 cm) and the gel was sealed with 1% agarose [29]. Electrophoresis was performed at 16 mA/gel for the initial 30 min and then at 24 mA/gel at 14°C until the running dye reached the bottom. The gel was stained with colloidal G-250 CBB (Biosafe; BioRad) and images were acquired by the gel imaging and spot picking system (Investigator™ ProPic, Genomic solution, USA). Well resolved protein spots of interest in the molecular weight range of 68 to 97.4 kDa (F2 fraction) were excised by hand (confirmed by rescanning the gel). The in-gel digestion of proteins and purification of peptides from plugs was carried out according to the manufacturer's manual. Briefly, protein spots were excised, washed with desalted water, followed by 50% v/v ACN in 25 mM ammonium bicarbonate (pH 8.0), shrunk by dehydration in ACN and vacuum dried. Gel pieces were reswollen in 10–20 μL digestion buffer containing sequencing grade modified 10 μg/mL trypsin (Promega, Madison, WI, USA). After 15 min, 25 μL of 50 mM ammonium bicarbonate was added to keep the gel pieces wet during tryptic cleavage (37°C, overnight). To extract the peptides, 50% ACN/0.3%TFA solution was added, and the samples were incubated for 15 min and vortexed. The separated liquid was dried under vacuum and the peptides were again dissolved in 10 μL 0.1% TFA. The peptides were purified with C18 reversed-phase minicolumn filled in a micropipette tip, ZipTip C18 (Millipore, Bedford, MA, USA), before MS. The peptide solution was then mixed with a double volume of matrix, CHCA (ABI, Farmingham, USA.) of 10 mg/mL in 50% ACN, 0.1% TFA and spotted onto a MALDI sample plate.

MS and MS/MS spectrum were acquired in the positive ion mode on MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Framingham, MA, USA). The instrument was operated in the delayed extraction mode with delay time of 200 ns. Spectra were obtained by accumulation of 1000 and 4000 consecutive laser shots respectively in MS and MS/MS mode and laser intensity used were in the range of 5000 to 6000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems) a standard mixture of six peptides des-Arg1-Bradykinin (904.4681), Angiotensin I (1296.6853), Glu1-Fibrinopeptide B (1570.6774), ACTH [clip 1–17] (2093.0867), ACTH [clip 18–39] (2465.1989) and ACTH [clip 7–38] (3657.9294). Mass calibration for MS/MS spectra was performed by fragment masses of precursor Glu1-Fibrinopeptide B (1570.6774). Peak harvesting was carried out using 4000 Series Explorer™ Software (Applied Biosystems). Only baseline corrections were applied to the raw data.

Database searching for protein identifications was performed with mass spectrometry data (MS or MS/MS) using Global Proteome Server v3.5 software (Applied Biosystems) equipped with MASCOT (Matrix Science) search engine. Only monoisotopic masses were used for searching against the Swiss-prot and NCBInr databases with a minimum number of matched masses set at 4. The maximum peptide

precursor tolerance was set at 40 ppm and MS/MS fragment tolerance was defined as 0.2 kDa. At the most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification and methionine oxidation as variable modification. MS/MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF-MS data. The criteria used to accept identifications for PMF included the probabilistic protein score-based confidence interval percentage, the extent of sequence coverage, the number of peptides matched and whether any *Leishmania sp*. or *Trypanosoma* protein appeared as top candidates during the first search, when no restriction was applied to the species of origin. Identification criteria with MS/MS data were that peptide count should be not less than two or more and confidence interval percentage for the best ion score should be above 95.

SLD from the *Leishmania* was separated by high-resolution 2-DE as shown in Fig.1. The gels prepared with preparative protein loadings (1.5 mg) displayed good resolution with only minimal streaking. CBB staining showed several abundant protein groups and many other minor components suitable for MS analysis were also detected. Though silver staining might have detected more spots, it could have posed problems during the process of protein identification by MALDI-MS. The reproducibility of the 2-D patterns was confirmed and considered final when two consecutive runs produced identical patterns. In total, 63 well resolved protein spots (Fig. 1) were detected in CBB stained gels with pH ranging from 3 to 10 as well as under the molecular mass ranging between 68 and 97·4 kDa. Majority of the proteins were detected around 4 to 8 p*I* acidic to neutral pH range. Experimental masses and p*I* were calculated using Progenesis software; measures of the confidence of the identification on the basis of number of peptides matched and sequence coverage was determined using MASCOT.

At present, ~33.6 Mb genome (8300 protein coding genes, http://www.genedb.org/leish/index.jsp) of *Leishmania major* has been sequenced [30]. In consequence, it was necessary to attempt to identify *L. donovani* proteins by reference all known *Leishmania* spp. sequences in the Swiss-Prot database. This strategy has been applied with success for other *Leishmania* species that lack a fully sequenced genome [31, 32]. The proteins thus identified are listed in detail in Table 1. In all, 63 well resolved protein spots were excised from a CBB stained gel for analysis and the data was

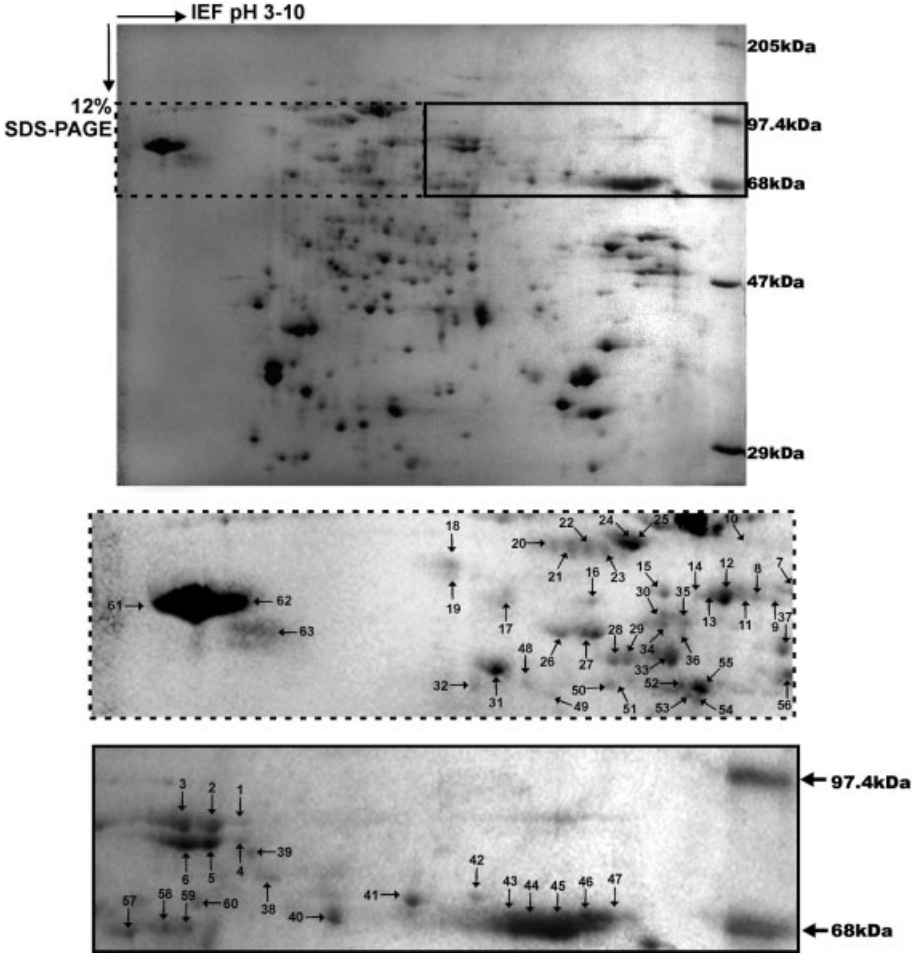

Figure 1. 2-D image of soluble *Leishmania* extract as well as respective partial enlarged 2-D gels of F2 (68–97.4 kDa) fraction. The total protein extracts (1.5 mg) were separated on linear IPG strips (pH 3–10) followed by 12% SDS-PAGE. The gel was stained with CBB. Numbers indicate protein spots that have been processed for MALDI.

Table 1. Proteins identified in the range of 68–97.4 kDa (F2 fraction) of the soluble *L. donovani* promastigote antigen

SN[a]	Identified Proteins[b]	Sp.[c]	Acc. No[d]	kDa/pI[e]	Pm/Ms/Sc%[f]	FC[g]	Remarks and Ref[h]
1	Fructose-1,6-bisphosphatealdolase	*Lmx*	5834626	47/7.9	14/123/24	1–3	VC, DT[58–61]
2	Fructose-1,6-bisphosphatealdolase	*Lmx*	5834626	47/7.5	16/155/38	1–3	VC, DT[58–61]
3	Fructose-1,6-bisphosphatealdolase	*Lmx*	5834626	41/7.0	16/156 /32	1–3	VC, DT[58–61]
4	Elongation factor-2	*Lmj*	11244578	73/7.2	15/114 /29	4	Th1 [38]
5	Fructose-1,6-bisphosphatealdolase	*Lmx*	5834626	45/7.9	14/119/25	1–3	VC, DT[58–61]
5	Probablyexpressed; ORFA; putative	*Li*	507273	66/6.8	12/57/41	?[i]	?[i]
6	Fructose-1,6-bisphosphate aldolase	*Lmx*	5834626	41/7.5	12/145/25	1–3	VC, DT[58–61]
7	DisulfideisomerasePDI	*Lmj*	25990151	52/5.2	8/100/21	1	VF,DT,VC[55–57]
8	Triosephosphate isomerase, glycosomal	*Tc*	TPIS_TRYCR[j]	76/6.6	13/232/18	1–3	Th1,VC[59–63,64–66]
9	Adenosylhomocysteinase	*Ld*	1710837	48/5.7	16 /148/21	5	DT[71]
9	p45	*Lmj*	6274526	41/6.6	11/96 /46	4	T cell st[38]
10	Hypothetical protein	*Tb*	25992877	76/5.7	7/125/58	??[k]	?[i]
11	Adenosylhomocysteinase	*Ld*	1710837	48/5.8	13/106/21	5	DT[71]
12	Enolase	*Lmj*	8388689	46/5.6	9/62/34	1–4	IGP[59–61,67–70]
13	Enolase	*Lmj*	8388689	46/6.6	16/167/27	1–4	IGP[59–61,67–70]
14	DisulfideisomerasePDI	*Lmj*	25990151	52/5.2	9/100/25	1	VF,DT,VC[55–57]
15	Protein of unknown function	*Tc*	32401138	38/5.22	15/121/36	??[k]	?[i]
16	Calreticulin	*Lmj*	5263289	33/4.7	8/67/29	1	VF,IGP[51–54]
16	Hypothetical protein L2385.08	*Lmj*	12311835	81/4.4	15/79/35	??[k]	?[i]
17	Hypothetical protein, unlikely	*Tb*	25992853	53/5.5	12/67/32	??[k]	?[i]
20	Heat shock 70-related protein1 precursor	*Lmj*	50299857	69/5.5	14/105/36	1	Th1 [39–50]
21	DnaK-type molecular chaperone hsp70.4	*Lmj*	7441842	70/5.5	13/89/39	1	Th1 [39–50]
22	Heat shock 70-related protein1 precursor	*Lmj*	50299857	69/5.5	9/128/29	1	Th1 [39–50]
23	Heat shock protein-90	*Ld*	323030	53/5.6	14/109/33	1	Th1 [39–50]
24	Hsp83 protein	*Ldi*	362545	81/5.1	14/71/21	1	Th1 [39–45, 49, 50]
24	Hypothetical protein L2385.08	*Lmj*	12311835	84/5.4	11/92/26	??[k]	?[i]
25	Cofactor-independent phosphoglycerate mutase	*Lmx*	28400787	61/5.4	9/177/23	5	DT[77]
26	Cofactor-independent phosphoglycerate mutase	*Lmx*	28400787	61/5.4	11/114/22	5	DT[77]
27	Trypanothionereductase	*Lmj*	7677022	53/5.8	13/115/37	5	DT[72]
28	Trypanothionereductase	*Lmj*	7677022	53/5.8	15/120/12	5	DT[72]
29	Adenosylhomocysteinase	*Ld*	1710837	48/5.7	15/130/23	5	DT[71]
30	Adenosylhomocysteinase	*Ld*	1710837	48/5.7	10/72/23	5	DT[71]
31	Hypothetical protein L5769.02	*Lmj*	12311865	29/6.8	9/84/28	??[k]	?[i]
32	Hypothetical protein L5769.02	*Lmj*	12311865	29/6.8	11/86/34	??[k]	?[i]
33	probablet-complex protein1, delta subunit	*Lmj*	9857048	60/6.7	15/83/22	??[k]	?[i]
34	Hypothetical protein	*Lmx*	2131001	48/7.1	11/68/22	??[k]	?[i]
35	Hypothetical protein	*Lmx*	2131001	45/7.1	9/35/19	??[k]	?[i]
36	Enolase	*Lmj*	8388689	46/6.6	16/104/27	1–4	IGP[59–61,67–70]
40	Glutamate dehydrogenase	*Tc*	3080751	49/6.5	16/116/26	5	DT[73]
42	Proteinphosphatase-2C	*Ld*	2665676	45/7.8	21/230/39	3	DT[78]
43	Proteinphosphatase-2C	*Ld*	2665676	45/7.8	23/261/38	3	DT[78]
44	Proteinphosphatase-2C	*Ld*	2665676	45/7.8	21/119/25	3	DT[78]
45	Proteinphosphatase-2C	*Ld*	2665676	45/7.8	23/79/34	3	DT[78]
49	Pyruvate kinase	*Lmx*	577072	55/6.0	11/92/28	3	DT[74]
50	Pyruvate kinase	*Lmx*	577072	55/6.0	11/94/26	3	DT[74]
51	Pyruvate kinase	*Lmx*	577072	55/6.0	15/143/27	3	DT[74]
51	Dihydrolipoamide dehydrogenase	*Lmj*	44804791	51/6.4	11/125/15	3	DT[79]
52	Pyruvate kinase	*Lmx*	577072	55/6.0	11/119/27	3	DT[74]
52	Dihydrolipoamide dehydrogenase	*Lmj*	44804791	51/6.4	10/63/29	3	DT[79]
54	Dihydrolipoamide dehydrogenase	*Lmj*	44804791	51/6.4	15/57/35	3	DT[79]
55	ChainG, of Pyruvate Kinase	*Lmx*	3660268	55/6.1	16/149/13	3	DT[74]
56	Cell division control protein-2 -homolog-1	*Tc*	CC2H1_TRYCO[j]	34/7.7	14/76/29	4	DT[80]
58	NADP-dependent alcohol hydrogenase	*Lmj*	6066457	39/5.8	11/187/23	5	?[i]
59	Hypothetical protein L236.01	*Lmj*	6580519	79/8.0	12/84/21	??[k]	?[i]
60	Kinesin-like protein K39	*Ldc*	KINL_LEICH[j]	76/4.2	9/93/31	1	IDP [75]

Table 1. Continued

SN[a)]	Identified Proteins[b)]	Sp.[c)]	Acc. No[d)]	kDa/p*I*[e)]	Pm/Ms/Sc%[f)]	FC[g)]	Remarks and Ref[h)]
61	Hypothetical protein L2385.08	*Lmj*	12311835	145/4.5	15/77/26	??[k)]	?[i)]
62	Heat shock 70-related protein1 precursor	*Lmj*	50299857	70/5.5	14/105/29	1	Th1 [39–50]
63	NAD-dependent deacetylase SIR2 homolog	*Lmj*	SIR2_LEIMA[j)]	43/5.64	12/63/17	5	DT[76]

The protein spots indicated in Fig. 1 were identified using PMF.

a) Protein spots no. indicated in Fig. 1

b) Name of the protein

c) Species: Lmx, *L. mexicana*; Lmj, *L. major*; Li, *L.infantum*; Ld, *L. donovani*; Ldi, *Leishmania. donovani Infantum*; Ld, *L. donovani chagasi*; Tb, *T. brucei*; Tbr, *T. brucei rhodesiense*; Tc, *T. Cruzi*.

d) Accession numbers according to NCBI and Swiss-Prot accession number

e) Experimental M_r and p*I*

f) No. of Peptides matched/MOWSE score/Sequence covered percentage

g) Identified proteins fell into the following major five functional categories; with some of them falling into two or more groups: 1. Stress response; 2. Cytoskeleton and cell membrane; 3. Energy metabolism and phosphorylation; 4. Cell cycle and proliferation; 5. Amino acid metabolism.

h) Remarks and References: VC; Vaccine candidate, Th1; Th1 Stimulatory, T cell st; T cell stimulatory proteins, VF; Virulence factor, DT; Drug target molecule, IGP; Immunogenic protein, IDP; Immunodiagnostic protein. Note: Protein spots analyzed but not identified: 18, 19, 37, 38, 39, 41, 46, 47, 48, 53, and 57.

i) Not previously described

j) Swiss-Prot accession number

k) Unknowns/hypothetical function of the protein not known.

retrieved for 52 (82·5%) spots by MS and MS/MS analysis. The identified spots matched to 168 database entries. Of spots analyzed by MALDI-TOF and MS/MS, 41% were clearly identified by their homology with those of *L. major*. Minor identification failures could be due to sample amount, specific peptide characteristics and extensive PTMs or significant divergence from sequenced strains [33]. In all, a total of 33 proteins were identified including six hypothetical proteins/unknowns. Among these, major proteins were of known immunostimulatory or immunogenic type or have been evaluated as vaccine candidates such as elongation factor-2, p45, heat shock protein (HSP) 70, HSP83, fructose-1, 6-bisphosphatealdolase, enolase, triosephosphate isomerase, protein disulfideisomerase, calreticulin, kinesin-like protein (K39). Some of the other proteins including some enzymes from energy metabolism, phosphorylation pathway, amino acid metabolism pathways and from diverse metabolic routes have also been reported as potential drug targets *viz*. fructose-1, 6-bisphosphatealdolase, enolase, triosephosphate isomerase, protein disulfideisomerase, proteinphosphatase-2C, pyruvate kinase, dihydrolipoamide dehydrogenase, adenosylhomocysteinase, cofactor-independent phosphoglycerate mutase, trypanothione reductase, glutamate dehydrogenase, and NAD-dependent deacetylase SIR2 homolog.

In addition, we have also observed that the mass and charge of several proteins were different from those predicted by the leishmanial genome, which is hitherto reported to be a common feature of most proteomic analyses, probably reflecting the effect of protein 'maturation' events including co- or PTM [23, 33]. Further, in our study we have detected more than one protein in a single spot for example, pyruvate kinase and dihydrolipoamide dehydrogenase, *etc*. (some more examples are listed in Table 1). In the same way, many proteins appeared as more than one spot representing their multiple 'charge' and/or 'mass' forms like aldolase, enolase, HSP83, pyruvate kinase, adenosylhomocysteinase. These observations suggest that PTM is widely prevalent in this organism. Similarly, in other studies of *Leishmania* also, predicted masses different from the observed masses have been seen where primarily tubulins and HSPs were found as multiple spots [13, 33]. Bente *et al* [13] have also supported the finding but attributed it due to degradation during sample preparation.

Although *Leishmania* is a well-studied pathogen that causes fatal VL, the SLD of *L. donovani* has remained unexplored so far. To date, analysis of the whole *Leishmania* proteome have been made by different research groups to understand the developmentally induced changes and studies related to identify proteins responsible for antigenicity [34] as well as for drug resistance mechanisms [12, 35–37]. However, this is the first study related to the characterization of immunostimulatory antigens from the F2 fraction of SLD proteins. The soluble leishmanial proteins could also be degraded into peptides, which, upon association with major histocompatibility complex class II molecules and presentation to $CD4^+$ T cells, trigger host cellular immune responses [38] hence confirming our views that SLD proteins may constitute vaccine candidates [8].

Most of the proteins that have been identified in the F2 fraction fall within five major categories defined by predicted function, such as (i) stress response; (ii) cytoskeleton assembly; (iii) energy metabolism and phosphorylation; (iv) cell

cycle control and proliferation; (v) amino acid metabolism. Interestingly, among the identified proteins in this range most noteworthy was the presence of the heat-shock proteins such as HSP70, dnaK type HSP70, HSP83, HSP90 (synonym of HSP83). Emerging evidence indicates that HSPs are a set of highly evolutionary conserved proteins. High-molecular-weight HSPs participates in a large number of biochemical and immunological pathways. They behave as chaperones [39–41], as immunodominant antigens [42] and are also implicated in the antigen-processing pathway [43, 44]. HSPs have been shown to act as immunostimulants of antigen-specific T cells [45] predominantly of Th1 characterized by induction of cytotoxic T lymphocyte activity and cytokine secretion, *e.g.* chimeric HSP70 stimulated strong immunostimulatory response in mammals [46] *M. tuberculosis* [47] and *L. infantum* [48]. *Leishmania* HSPs, mainly the HSP70 and HSP83 are known to potentiate a Thl-type response and their importance as potent immunogens in leishmaniasis have also been recognized [49]. Moreover, they have also been implicated in immunoprotection [50]. Among the other identified proteins elongation factors-2 and p45 (spot no 4 and 9. respectively) have been reported to induce proliferative response in cured VL patients peripheral blood mononuclear cells as well as leishmanial parasite-specific T cell lines derived from an immune donor [38].

Another interesting stress shock protein identified in the present study is calreticulin, a chaperonin involved in glycoprotein folding. It is an important multifunctional immunodominant calcium (Ca+2)-binding protein [51–53]. Calreticulin in *Leishmania* may affect the targeting of proteins that are associated with the virulence of the parasite [54]. Another protein responsible for virulence factor, identified in the present study, was protein disulfideisomerase an excreted/secreted antigen [55], which has been previously reported by Achour *et al.* [56]. Earlier work has shown that protein disulfideisomerase plays a potential role in the pathogenicity of various microorganisms. It is believed that protein disulfideisomerase represents a new potential component of novel immunogenic or vaccine preparations aimed at conferring immunity in humans or animals against *Leishmania* [57].

Some other proteins detected in MALDI analysis such as glycolytic enzyme fructose-1,6-bisphosphatealdolase (aldolase), enolase, and triosephosphate isomerase may also be considered as potential vaccine candidates, since they have been reported to be immunogenic in other organisms [58–70]. Aldolase is described as a potential vaccine candidate for the prevention of infection with the filarial nematode *Onchocerca volvulus* [58]. Extensive work has already been carried out with many glycolytic enzymes, like aldolase, enolase, triosephosphate isomerase of *Plasmodium falciparum,* which have also been identified in our study, that have showed promise as good vaccine candidates, diagnostic tools and drug targets [59–63]. Triosephosphate isomerase is considered as a potential vaccine against *Schistosoma mansoni* by inducing IL-2 and IFN-γ production (Th1 responses) [64–66]. Studies by Sundstrom and Aliaga [67] have reported enolase (spotted in this study) as an immunodominant antigen in an experimental model of *C. albicans* infection [68–70]. These glycolytic enzymes may be considered as novel parasite proteins as none of these have been reported previously as T cell-stimulating antigens from *Leishmania* and therefore, can be exploited as novel vaccine candidate against VL.

Notably, the proteomic study presented here also documents completely unknown or hypothetical proteins of the parasite, which may represent potential targets for drug development or putative vaccine candidates. Additional studies such as cloning and expression of the best antigenic targets, as determined by their immunoprotective potential, together with their specific association and definite allocation are needed to characterize these new proteins.

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