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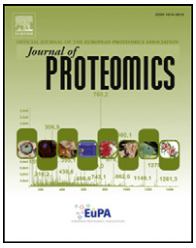

Serum profiling of leptospirosis patients to investigate proteomic alterations☆

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ABSTRACT

Leptospirosis is a zoonotic infectious disease of tropical, subtropical and temperate zones, which is caused by the pathogenic spirochetes of genus *Leptospira*. Although this zoonosis is generally not considered as fatal, the pathogen can eventually cause severe infection with septic shock, multi-organ failure and lethal pulmonary hemorrhages leading to mortality. In this study, we have performed a proteomic analysis of serum samples from leptospirosis patients (n=6), febrile controls (*falciparum* malaria) (n=8) and healthy subjects (n=18) to obtain an insight about disease pathogenesis and host immune responses in leptospiral infections. 2DE and 2D-DIGE analysis in combination with MALDI-TOF/TOF MS revealed differential expression of 22 serum proteins in leptospirosis patients compared to the healthy controls. Among the identified differentially expressed proteins, 8 candidates exhibited different trends compared to the febrile controls. Functional analysis suggested the involvement of differentially expressed proteins in vital physiological pathways, including acute phase response, complement and coagulation cascades and hemostasis. This is the first report of analysis of human serum proteome alterations in leptospirosis patients, which revealed several differentially expressed proteins, including α-1-antitrypsin, vitronectin, ceruloplasmin, G-protein signaling regulator, apolipoprotein A-IV, which have not been reported in context of leptospirosis previously. This study will enhance our understanding about leptospirosis pathogenesis and provide a glimpse of host immunological responses. Additionally, a few differentially expressed proteins identified in this study may further be investigated as diagnostic or prognostic serum biomarkers for leptospirosis.
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1. Introduction

Leptospirosis is caused by a Gram-negative like, obligate, aerobic spirochete of genus *Leptospira*, which is sustained in nature within a wide range of reservoir and carrier animals, including rodent and non-rodents. It is a contagious disease, transmitted through water or soil contaminated with urine of animal hosts containing a large number of this pathogenic spirochete. This zoonosis generally appears as a seasonal infection that initiates at the beginning of monsoon, occurs frequently during the rainy season and disappears when the rains recede; except few sporadic cases that may happen throughout the year [1,2]. Due to the increasing number of severe leptospirosis infections in developed and developing countries, it has been recognized as a major health problem [3]. Additionally, this emerging infectious disease can adversely affect agricultural industry [4]. Consequently, this fatal infectious zoonosis has a devastating impact on human health and corresponding impediment to economic improvement worldwide.

Leptospirosis is a systemic disease of humans and broad range of feral and domestic animals, which is characterized by fever, myalgia, conjunctival suffusion, renal and hepatic insufficiency, pulmonary manifestations and reproductive failure [5]. Renal involvement is very common in leptospirosis patients and severe cases often lead to acute renal failure. Pulmonary alveolar haemorrhage is the prime cause of leptospirosis related casualties [6]. Successful completion of the genome sequence of *Leptospira interrogans* [7] has paved the emergence of omics-based approaches in leptospirosis research [8]. Proteome level analysis of different biological fluids has been found to be effective for investigation of disease pathobiology as well as identification of surrogate markers with diagnostic and prognostic significance [9,10]. Recent proteomic studies have generated valuable information regarding the global/differential proteome and immunome profile of pathogenic *Leptospira* spp. [11–13], protein expression and multiple modification system of the pathogen [14], and analysis of leptospires excreted in urine of chronically infected hosts [15,16]. To this end, notable achievements have been accomplished in deciphering the global and sub-cellular proteome profile of this pathogenic spirochete using quantitative mass spectrometric approaches [17,18]. However, the molecular pathogenesis of leptospirosis is not clearly understood and requires further comprehensive studies at the molecular levels. The omics-based investigations can reveal the mechanism of disease pathogenesis and the pathogen induced alterations in host physiological system as well as identification of leptospiral immunogens useful for diagnostic applications and vaccine development.

In the present study, we have performed a comprehensive proteomic analysis of serum samples from patients suffering from leptospirosis using two dimensional gel electrophoresis (2DE) and 2D fluorescence difference gel electrophoresis (2D-DIGE), and compared with febrile (*falciparum* malaria) and healthy controls. Identification of differentially expressed proteins was performed using high-sensitivity MALDI-TOF-TOF mass spectrometry. In this differential proteomics analysis, we have identified 22 statistically significant ($p<0.05$) proteins in leptospirosis patients compared to the healthy subjects. Proteins showing differential expression in leptospirosis patients were subjected to further functional clustering by using database for annotation, visualization and integrated discovery (DAVID) and protein analysis through evolutionary relationships (PANTHER). The possible involvement of differentially expressed proteins in various physiological pathways and the host immune response against the pathogen has been studied. As far our knowledge goes, this is the first report of human serum proteome alterations in leptospiral infection and our results provide valuable insight into the host immune response against this acute bacterial infection and the underlying molecular mechanisms of the disease pathogenesis.

2. Materials and methods

2.1. Patient selection and study design

Blood samples were collected from leptospirosis patients enrolled in Seth GS Medical College & King Edward Memorial hospital, Mumbai with approval of the institutional ethics committee and written informed consent from each subject. Leptospiral infection was diagnosed by clinical symptoms and confirmed through rapid test for IgM antibodies to *Leptospira* using Leptocheck WB kit. Symptomatic patients with 2–4 days of fever and positive rapid immunodiagnostic test having no past history of autoimmune diseases or any significant systemic diseases were selected for this study. Demographic, epidemiological and clinical details, including past history of diseases of all patients (n=6) selected for this proteomic study were recorded (Table S1.1). Blood specimens were also collected from age and sex matched healthy (n=18) (voluntary blood donors) and febrile controls (n=8) with written informed consent to perform comparative analysis. Patients with non-severe, uncomplicated, *falciparum* malaria diagnosed by microscopic examination and confirmed by rapid diagnostic tests (RDT) were selected as febrile controls (FC) for this proteomic study (Table S1.2). Serum separation tubes (BD Vacutainer®; BD Biosciences) were used to collect blood samples from the antecubital vein of the subjects. Subsequent to blood collection, samples were allowed to clot by keeping the tubes in ice for 30 min. After clotting, the samples were centrifuged at 2500 rpm at 20 °C for 10 min to separate serum from the clotted blood. Collected serum samples were labeled and stored in multiple aliquots at −80 °C. To minimize any pre-analytical variations, exactly similar collection, processing and storage conditions were maintained for each control and diseased samples.

2.2. Processing of serum samples, 2DE and 2D-DIGE

All the 32 samples [6 leptospirosis; 8 febrile controls (*falciparum malaria*) and 18 healthy subjects] were analyzed by classical 2DE in two technical replicates. A sub-set of 18 samples [6 each for leptospirosis (L), FC and HC] were analyzed individually by 2D-DIGE. Six representative subjects were selected from each group for 2D-DIGE analysis keeping the age range, median age value, sex, past history of systemic disease etc. in consideration. Sample processing for 2DE and 2D-DIGE were performed as

previously described [19]. In brief, a combination of TCA-acetone protein precipitation, sonication, albumin and IgG depletion and desalting was used for processing serum samples from leptospirosis patients and controls for proteomic analysis. Depletion of albumin and IgG, and desalting of the serum samples were performed using Albumin & IgG Depletion SpinTrap (GE Healthcare) and 2D Clean-up kit (GE Healthcare), respectively, following the manufacturer's instructions. After processing of protein extracts, the serum proteins were dissolved in rehydration buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4–7; Linear), 40 mM DTT and traces of bromophenol blue. Prior to the 2DE/2D-DIGE experiment protein concentration in each sample (leptospirosis patients and controls) was estimated using the 2D-Quant kit (GE Healthcare) following the manufacturer's instructions. The 600 μg of protein was loaded on 4–7 pH range, 18 cm IPG strips, and isoelectric focusing was performed for overall ~78 kVh using Ettan IPGphor 3 isoelectric focusing unit (GE Healthcare). The second dimension was performed on 12.5% SDS polyacrylamide gels in an Ettan DALTsix electrophoresis unit (GE Healthcare) and Gel Code Blue Safe Protein Stain (Thermo Scientific, USA) was used for the visualization of the protein spots. In order to test the reproducibility and reduce technical artifacts, every sample was analyzed in duplicate. In 2D-DIGE, test and control samples (leptospirosis patients and HC/FC) were labeled with Cy3 and Cy5, while an equal amounts (total serum proteins) of all samples (L, FC and HC) to be analyzed in the experiment; regarded as internal standard was labeled using the third fluorescent dye, Cy2, following the manufacturer's instructions (GE Healthcare). While labeling of test and control samples, the dye swapping was performed to eliminate any type of dye effects. Subsequently, IEF and SDS-PAGE separation were performed following the same protocol as employed in the regular 2DE experiment.

2.3. Image acquisition and software analysis

The 2D gel images were scanned by using LabScan software version 6.0 (GE Healthcare) and analysis was performed by using ImageMaster 2D Platinum 7.0 software (GE Healthcare). 2D-DIGE gels were scanned using Ettan DIGE Imager scanner (GE Healthcare) using proper wavelengths and filters for Cy2, Cy3 and Cy5 dyes keeping the resolution at 40 μm. Accurate cropping of the gel images were performed using ImageQuant software version 5.0 (GE Healthcare). The cropped gel images were imported to ImageMaster 2D Platinum 7.0 DIGE software (GE Healthcare) and subjected to comparative analysis for relative protein quantification across all the leptospirosis and control samples. The average ratio of expression was analyzed by Student's t-test. Protein spots showing differential expression with reproducibility and statistical significance ($p<0.05$) were selected and excised for further MS analysis.

2.4. In-gel trypsin digestion and mass spectrometry

Protein spots identified in 2DE and 2D-DIGE gels exhibiting differential expression with statistical significance ($p<0.05$) in leptospirosis patients compared to the healthy subjects were excised for in-gel digestion and further MS analysis. In-gel digestion of the proteins was performed as described previously [19]. The identity of differentially expressed proteins was established using AB Sciex 4800 MALDI-TOF/TOF mass spectrometer. The combined MS and MS/MS peak lists were searched using the GPS™ Explorer software version 3.6 (AB Sciex). The MASCOT version 2.1 (http://www.martixscience.com) was used as the search engine for protein identification against the Swiss-Prot database. During the database search following parameters were specified: human taxonomy, trypsin digestion with one missed cleavage, carbamidomethyl (C) as fixed modification, oxidation (M) as variable modification, peptide mass tolerance set at 75 ppm and MS/MS tolerance of 0.4 Da.

2.5. Western blot analysis

Western blot analysis was performed with serum samples from controls (healthy and febrile) and leptospirosis patients (n=6) to validate the differential expression of some of the target proteins identified in 2DE and 2D-DIGE experiments. Prior to the western blotting experiment protein concentration in each sample (leptospirosis patients and controls) was estimated using the 2D-Quant kit (GE Healthcare) and BCA Protein Assay kit (Thermo Fisher Scientific) following the manufacturer's instructions. The serum proteins were separated on a 12% SDS-PAGE (50 μg of total proteins per lane) and then transferred onto PVDF membranes under semi-dry conditions by using an ECL semi-dry transfer unit (GE Healthcare). Protein migration was tracked by using pre-stained protein standard (Fermentas). Western blotting was performed by using monoclonal/polyclonal antibody against clusterin (Santacruz biotechnology, sc-8354), ceruloplasmin (Santacruz biotechnology, sc-365206) and appropriate secondary antibody conjugated with HRP (GeNei (MERCK)-621140380011730 or 621140680011730). ImageQuant software version 5.0 (GE Healthcare) was used for quantitation of the signal intensity of the bands in western blots.

2.6. Protein networks and functional analysis

The differentially expressed proteins in leptospirosis patients were subjected to functional pathway analysis using PANTHER software, version 7 (http://www.pantherdb.org) [20] and DAVID database version 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) [21] for better understanding of the biological context of the identified proteins, their connection with disease pathobiology and participation in various physiological pathways. UniProt accession numbers of the 22 differentially expressed proteins identified in our study were uploaded and mapped against the *Homo sapiens* reference dataset to extract and summarize functional annotation associated with individual or group of genes/proteins and to identify gene ontology terms, molecular function, biological process and important pathways for each dataset.

3. Results

3.1. 2DE and 2D-DIGE analysis of serum proteome in leptospiral infections

This comparative proteomic profiling was performed to identify differentially expressed serum proteins in leptospiral infection. Serum proteome profile of healthy subjects, febrile

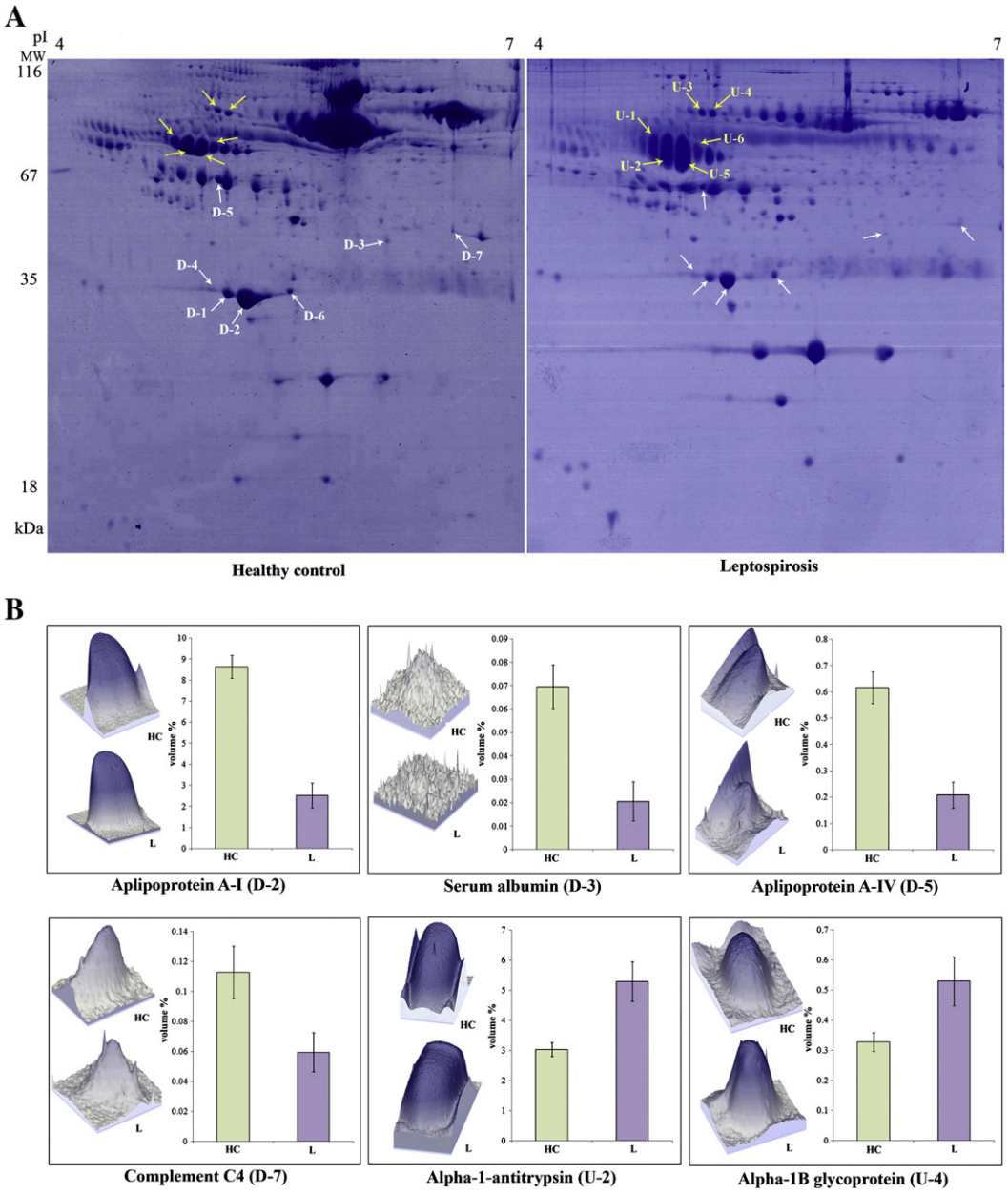

Fig. 1 – Differential expression of serum proteins in leptospirosis patients identified in 2DE analysis. (A) Representative 2D gels of serum proteome from healthy controls (n = 18) and leptospirosis patients (n = 6) containing 600 μg depleted serum proteins. Protein samples focused on linear pH 4–7 IPG strips (18 cm) and subsequently separated on 12.5% polyacrylamide gels, were stained with Gel Code Blue Stain. The statistically significant ($p < 0.05$) down (D) and up-regulated (U) protein spots are marked on 2D gels. (B) The 3D views and bar-diagrammatic representation of few statistically significant down and up-regulated serum proteins identified in patients suffering from leptospiral infection. Data is represented as mean ± SE.

controls and leptospirosis patients were analyzed using two gel-based proteomic platforms classical 2DE and advanced 2D-DIGE. In each analysis individual samples were studied (n = 32 for classical 2DE and n = 18 for 2D-DIGE) and sample pooling was not executed since it cannot reflects the true pictures of biological variability effectively. Differentially expressed protein spots identified in gel-based analysis, which full-filled the statistical parameters (t-test; $p < 0.05$) were subjected to further mass spectrometric and functional analysis.

Over 600 protein spots were detected in each 2D gel stained with GelCode Blue Safe Protein Stain using IMP7 software. 13 statistically significant ($p < 0.05$) differentially expressed, 7 down-regulated and 6 up-regulated (with fold changes ranging from −4.39-fold to +2.7-fold) proteins were identified in 2DE analysis (Fig. S1 and Table S2). Among the 6 up-regulated spots, 5 spots were between 1.1 and 2-fold, and 1 spot exhibited over 2-fold increase in expression level; while in the case of down-regulation, 2 spots were between 1.1 and 2-fold, 1 spot was between 2- and 3-fold, and 4 spots found to have over

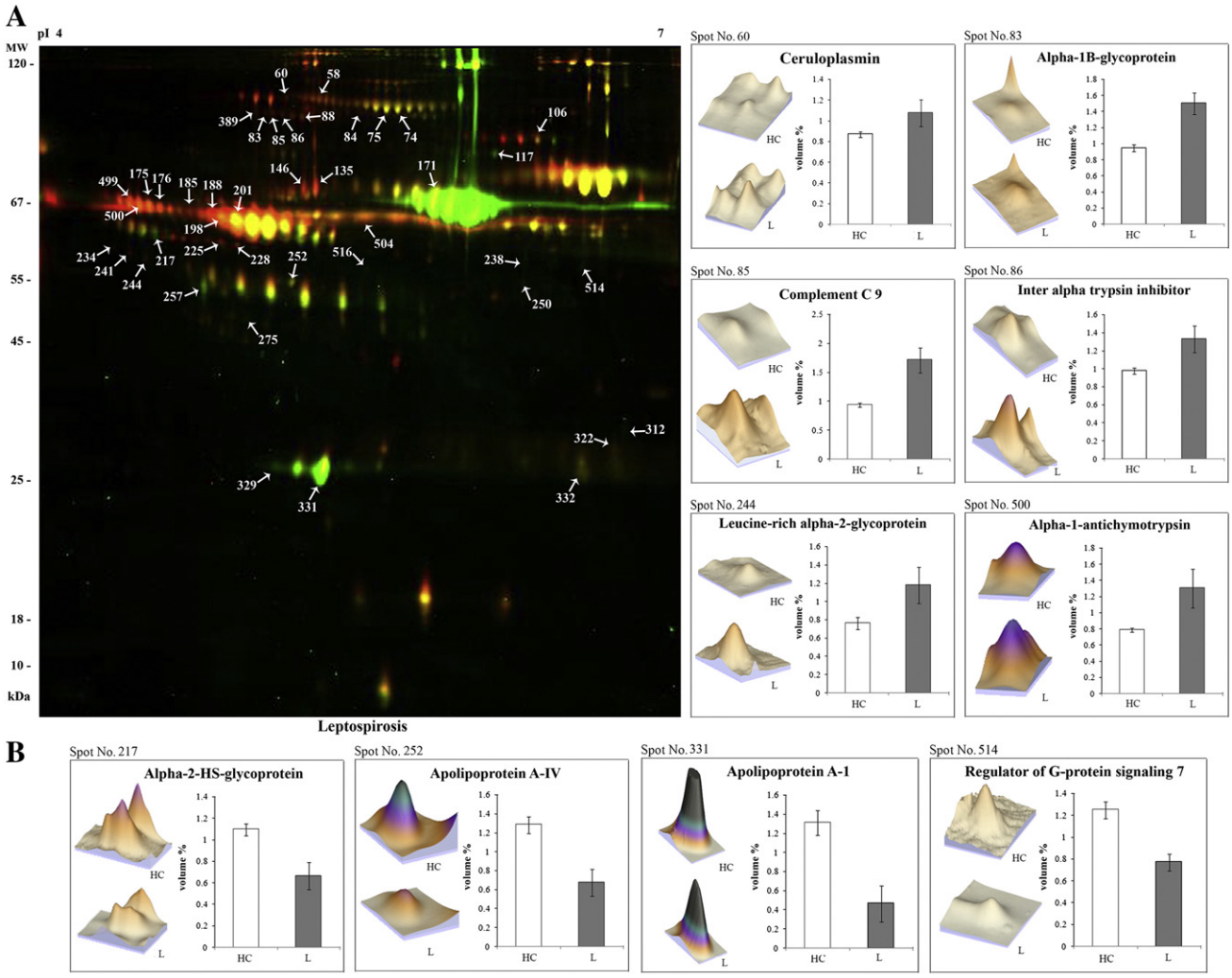

Fig. 2 – Differential expression of serum proteins in leptospirosis patients identified in 2D-DIGE analysis. (A) Representative 2D-DIGE image of proteins from healthy controls (HC) and leptospirosis patients (L). HC and leptospirosis samples were labeled with Cy3 and Cy5 respectively, while the protein reference pool (internal standard) was labeled with Cy2. (B) Bar-diagram and 3D views of fluorescence intensity of few selected statistically significant ($p<0.05$) differentially expressed proteins identified in leptospirosis patients.

3-fold reduced expression levels. Representative 2DE images of serum proteome profile of leptospirosis patients and healthy individuals, and bar-diagrammatic representation of the fold change and 3D views of some selected differentially expressed proteins are shown in Fig. 1A and B.

Second level of gel-based proteomic analysis was performed using 2D-DIGE approach, where the test (leptospirosis) and control (healthy/febrile) samples, after pre-electrophoresis labeling with CyDyes (GE Healthcare), were separated on the same gel to reduce gel-to-gel variations. Additionally, superior sensitivity of CyDyes compared to routinely used CBB stains, allowed to visualize less abundance protein spots in 2D-gels, which were not detectable in classical 2DE profiling, and thereby increased the overall coverage of serum proteome. Approximately 1000 protein spots were detected on each 2D-DIGE gels in the Image Master 2D Platinum 7.0 DIGE software analysis. In 2D-DIGE profiling, total of 98 (around 10% of the total detected spots) differentially expressed spots satisfied the statistical criteria (*t*-test and 1-way ANOVA; $p<0.05$), among which, 48 were up-regulated (range from 1.15 to 2.71-fold) while, the remaining 50 protein spots showed reduced expression level (with changes from 1.19 to 7.5-fold) in the leptospirosis patients (Table S3). 37 up-regulated spots were between 1.1 and 2-fold change range, and for the remaining 11 spots the range was between 2 and 3-fold; while among the 50 down-regulated spots, 33 were between 1.1 and 2-fold, 12 were between 2 and 3-fold, and 5 spots exhibited over 3-fold reduced expression level. Owing to the superior sensitivity and reproducibility in DIGE technique, we obtained much higher numbers of differentially expressed protein spots from 2D-DIGE experiment (Fig. 2A). Fig. 2B depicts 3D views and graphical representation of few selected differentially expressed protein spots.

3.2. *Identification of differentially expressed proteins using MALDI-TOF/TOF MS*

Alteration of human serum proteome due to the leptospiral infection was reflected by the differential expression of multiple

Table 1 – List of significant differentially expressed proteins identified in the Leptospirosis patients using 2DE.

Sl no.	Name of protein with UniProt accession number	Gene name	Fold change	MW (kDa)	No of spots (spot numbers)	*p* value (t-test)	Protein score[#]	Total ion score[#]	No. of matched peptides[#]	Functions[*]
Down-regulated proteins										
1	(P02647) Apolipoprotein A1 precursor	APOA1	4.39–1.91	30.75	4 (D-1,D-2, D-4,D-6)	2.30E−07	815	628	24	A,H,I
2	(P02768) Serum albumin precursor	ALB	3.4	69.32	1 (D-3)	0.00018	456	386	30	B,D,E,P,Q
3	(P06727) Apolipoprotein A-IV precursor	APOA4	2.96	45.34	1 (D-5)	5.80E−05	56	–	16	A,C,D,H,I
4	(P01028) Complement C4 precursor	C4	1.90	192.65	1 (D-7)	0.02288	492	483	19	J,L
Up-regulated proteins										
5	(P01009) Alpha-1-antitrypsin precursor	SERPINA1	2.71–1.47	46.71	4 (U-1,U-2, U-5,U-6)	0.0029	670	538	23	A,F
6	(P04217) Alpha-1B-glycoprotein precursor	A1BG	1.63–1.62	54.3	2 (U-3,U-4)	0.0405	900	821	16	G

[#]For proteins with multiple spots in the 2D gels, representative spot detail is provided. Exact values for each spot are provided in supplementary information.
[*]A—protein binding; B—DNA binding; C—metal binding; D—antioxidant activity; E—drug binding; F—peptidase/protease inhibitor activity; G—Plasma Glyco protein, function unknown; H—lipid binding; I—lipid metabolism/transport; J—brain development; K—enzyme regulator activity; L—complement activation; M—blood coagulation; N—antigen/antibody binding; O—catalytic activity; P—cell surface binding; Q—chaperone binding; R—enzyme inhibitor activity.

serum proteins in leptospirosis patients detected by gel-based profiling. Among the several differentially expressed protein spots detected in 2DE, those 13 spots that satisfied the statistical criteria ($p<0.05$) were subjected to in-gel trypsin digestion and subsequent MALDI-TOF/TOF analysis to establish protein identity. MS and MS/MS analysis results revealed that 13 differentially expressed protein spots identified in regular 2DE experiment correspond to 6 proteins, among which 2 were up-regulated (α-1-antitrypsin and α-1B-glycoprotein precursor) and the remaining 4 were down-regulated (apolipoprotein A1 precursor, serum albumin precursor, apo-lipoprotein A-IV precursor, complement C4 precursor) (Table 1 and S4.1).

In case of 2D-DIGE, selected spots were excised manually from preparative 2D gels stained with GelCode Blue Safe Protein Stain (Thermo Scientific, USA) containing higher amount of serum protein. Among the 98 differentially expressed protein spots, 57 spots that could be excised from the gels were subsequently subjected to MS and MS/MS analysis, which successfully established the identity of 42 spots (Fig. 2A; Table S4.2). For the remaining spots we were unable to establish MS identity most likely due to the very low intensity of those spots and insufficient amount of detectable peptides, probably beyond the sensitivity limit of the instrument. The 42 protein spots identified by MS correspond to 21 (10 down-regulated and 11 up-regulated) differentially expressed proteins in patients suffering from leptospiral infection (Fig. S2; Table 2).

In this study, we have performed two levels of gel-based proteomic analysis; initially classical 2DE and latter more advanced 2D-DIGE technology using sub-sets of the patient and control (febrile and healthy) populations studied by 2DE. Almost all of the proteins (except complement C4 precursor) identified in 2DE were again identified in 2D-DIGE profiling and exhibited similar trends of differential expressions which enhanced the confidence level of our study. The number of identified proteins in 2DE and 2D-DIGE are 6 and 21 respectively, while 5 of the identified proteins (apolipoprotein A-I, serum albumin precursor, apolipoprotein A-IV, alpha-1-antitrypsin and alpha-1B-glycoprotein) found to be overlapping for 2DE and 2D-DIGE, and overall 22 differentially expressed proteins ($p<0.05$) have been identified in our study (Tables 1 and 2). Pre-electrophoresis labeling of protein samples with highly sensitive Cy dyes in 2D-DIGE attributed around 55% increase in spot number compared to the CBB stained 2DE gels, which certainly enhanced the overall coverage of the whole serum proteome. In 2D-DIGE experiment we obtained 16 more differentially expressed proteins including complement C3 precursor, clusterin precursor, complement factor H, regulator of G-protein signaling 7 (down-regulated) and vitronectin precursor, ceruloplasmin precursor, leucine-rich α-2-glycoprotein (up-regulated) (Table 2), which were not identified in classical 2DE due to lower sensitivity and reproducibility issues.

Differentially expressed serum proteins identified in leptospiral infection (compared to the healthy subjects) were further investigated in *falciparum* malaria patients (febrile controls). Around 60% of the identified proteins found to be commonly modulated in both of the infectious diseases. However, for most of the proteins levels of altered expression were found to be different in leptospirosis compared to malaria. Interestingly, 3 of the identified proteins exhibited opposite trend of differential expression in leptospirosis compared to

Table 2 – List of significant differentially expressed proteins identified in the Leptospirosis patients using 2D-DIGE.

Sl no	Name of protein with UniProt accession number	Gene name	Fold change	MW (kDa)	No of spots (spot numbers)	*p* value (t-test)	Protein score[#]	Total ion score[#]	No. of matched peptides[#]	Functions[*]
Down-regulated proteins										
1	(P02647) Apolipoprotein A-I precursor (Apo-AI)	APOA1	2.83–3.01	30.75	2 (329, 331)	0.00284	815	628	24	A,H,I
2	(P02768) Serum albumin precursor	ALB	1.76–3	69.32	3 (117,171, 516)	0.03566	1370	1213	25	B,D,E,P,Q
3	(P01871) Ig mu chain C region	IGHM	1.73–2.28	49.53	1 (238, 250)	0.00521	391	352	11	N
4	(P01024) Complement C3 precursor	C3	2.21	187.05	1 (257)	0.00123	207	165	24	J,L
5	(P10909) Clusterin precursor	CLU	2.16	52.46	1 (275)	0.00168	241	211	10	A,O
6	(P06727) Apolipoprotein A-IV precursor	APOA4	1.91	45.43	1 (252)	0.03608	56	-	16	A,C,D,H,I
7	(P02765) Alpha-2-HS-glycoprotein precursor (Fetuin-A)	AHSG	1.65	39.3	1(217)	0.0276	405	369	9	A,K,R
8	(P08603) Complement factor H precursor	CFH	1.27–1.34	143.71	2(74, 75)	0.04136	778	572	40	L
9	(P49802) Regulator of G-protein signaling 7	RGS7	1.62	75.79	1(514)	0.02774	28	–	12	A
10	(P01834) Ig kappa chain C region	IGKC	1.2–1.35	11.6	3 (312, 322, 332)	0.03339	213	191	4	N
Up-regulated proteins										
11	(P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor)	SERPINA1	1.81–2.29	46.7	5 (185, 198, 201, 225, 228)	0.0233	667	520	22	A,F
12	(P04004) Vitronectin precursor (Serum spreading factor) (S-protein)	VTN	2.26	54.27	1 (188)	0.01282	173	164	5	A
13	(P02750) Leucine-rich alpha-2-glycoprotein precursor	LRG1	1.54–2.05	38.15	3 (234, 241, 244)	0.04313	634	568	13	A
14	(P04217) Alpha-1B-glycoprotein precursor	A1BG	1.30–1.89	54.3	4 (83,135, 146, 389)	0.01302	900	821	16	G
15	(P02748) Complement component C9 precursor	C9	1.84	63.13	1 (85)	0.03195	73	73	11	L,M,O
16	(P01011) Alpha-1-antichymotrypsin precursor	SERPINA3	1.40–1.68	15.88	4 (175, 176, 499, 500)	0.01572	375	220	27	A,B,F
17	(P00450) Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase)	CP	1.19–1.59	122.13	3 (58, 60, 88)	0.02839	437	264	35	C,D,Q
18	(Q14624) Inter-alpha-trypsin inhibitor heavy chain H4 precursor	ITIH4	1.36	103.29	1 (86)	0.01552	230	153	21	F
19	(P00751) Complement factor B precursor	CFB	1.17	85.4	1 (106)	0.04961	694	513	30	A
20	(P02649) Apolipoprotein E precursor	Apo-E	1.43	36.13	1 (84)	0.00758	669	573	18	A,H
21	(P01876) Ig alpha-1 chain C region	IGHA1	1.15	37.63	1 (504)	0.02851	612	560	11	A,H

[#]For proteins with multiple spots in the 2D gels, representative spot detail is provided. Exact values for each spot are provided in supplementary information.

[*]A—protein binding; B—DNA binding; C—metal binding; D—antioxidant activity; E—drug binding; F—peptidase/protease inhibitor activity; G—Plasma Glycoprotein, function unknown; H—lipid binding; I—lipid metabolism/transport; J—brain development; K—enzyme regulator activity; L—complement activation; M—blood coagulation; N—antigen/antibody binding; O—catalytic activity; P—cell surface binding; Q—chaperone binding; R—enzyme inhibitor activity.

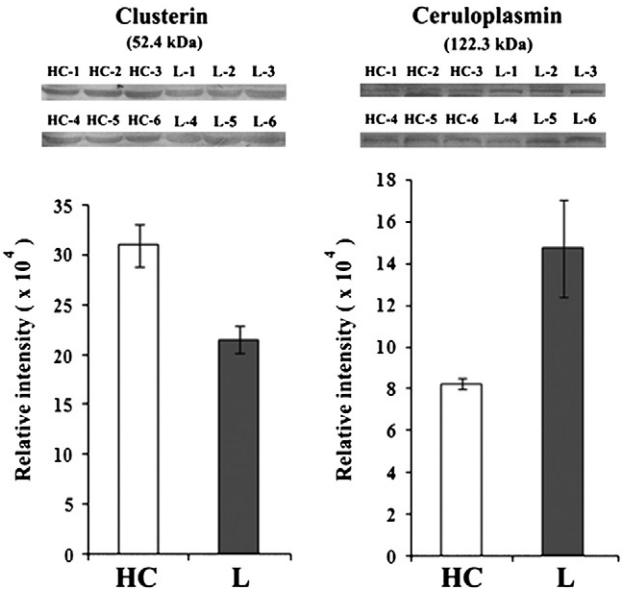

Fig. 3 – Validation of the differential expressions of clusterin and ceruloplasmin by western blot analysis. Western blot analysis of the target proteins: (A) clusterin and (B) ceruloplasmin are represented along with their respective relative intensity using bar-diagrams (relative intensity X 10^4). Data represents the mean ± SE for healthy controls (HC) (n = 6) and leptospirosis patients (L) (n = 6) analyzed in two independent technical replicates. Densitometry analysis of western blots revealed down-regulation of clusterin and up-regulation of ceruloplasmin in the leptospirosis patients compared to the healthy controls ($p < 0.05$) that confirmed the results obtained in 2D-DIGE experiment.

P. falciparum infection. Moreover, 5 proteins found to be differentially expressed in leptospiral infection, but not in febrile controls (Table S5). Bar-diagrammatic representation of altered expression levels of different serum proteins in leptospirosis and *falciparum* malaria has been depicted in Fig.S3.

3.3. *Validation of differentially expressed proteins*

Two selected differentially expressed proteins; clusterin and ceruloplasmin identified in leptospirosis patients were measured using western blotting to confirm the results of proteomic analysis. Equal loading of the samples was verified by CBB staining of the SDS-PAGE gels and Ponceau staining of the transferred blots containing the resolved proteins (Fig. S4A and B). Relative abundance of clusterin in healthy controls was found to be 245,347.6 ± 12,716.99, while the leptospirosis patients exhibited a mean value of 181,617.1 ± 12,369.32 (mean ± SE; $p < 0.05$; n = 6). In case of ceruloplasmin, the trend of differential expression found to be opposite; 154,517.4 ± 7561.17and 230,973.3 ± 37,899.88 in HC and leptospirosis patients, respectively. Western blot analysis revealed 1.35-fold down-regulation of clusterin (Fig. 3A) and 1.49-fold up-regulation of ceruloplasmin in the leptospirosis patients compared to the HC (Fig. 3B). Additionally, comparative analysis performed between leptospirosis patients and febrile controls (n = 6 each), revealed 1.1-fold down-regulation of clusterin and 1.13-fold up-regulation of ceruloplasmin in leptospiral infection compared to *falciparum* malaria (Fig. S4C and D). Western blot analysis of each sample (L, FC and HC) were performed in duplicate to check the reproducibility and minimize technical artifacts.

3.4. *Modulation of physiological pathways in leptospirosis studied by functional pathway analysis*

Functional pathway analysis was performed with 22 differentially expressed serum proteins identified in the leptospirosis patients using gel-based and MS-based analysis to understand their biological context, involvement in diverse physiological pathways and association with leptospiral infections. PANTHER analysis revealed involvement of identified proteins in blood coagulation system (33.3%), heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (33.3%) and heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (33.3%) (Table S6.1; Fig. 4A).

Concerning biological process, the identified proteins were involved in 10 biological processes: the major five processes include metabolic process (17.3%), immune system process (16%), response to stimulus (13.3%), cell communication (13.3%) and cellular process (13.3%), (Table S6.1; Fig. 4B). Five major GO functions: binding (31.4%), receptor activity (17.1%), enzyme regulator activity (22.9%), catalytic activity (11.4%) and transporter activity (17.1%) were identified in PANTHER analysis for the differentially expressed proteins in leptospirosis patients (Table S6.1; Fig. S5).

In DAVID analysis, KEGG category revealed complement and coagulation cascades (1.01 E^{-09}; 30.43%). Reactome category revealed three biological pathways: signaling in immune system ($p = 0.010079$; 21.74%), hemostasis ($p = 0.035589$; 17.39%), metabolism of lipids and lipoproteins ($p = 0.01712$; 17.39%), while, different complement cascades (Fig. 4C) including classical complement pathway ($p = 4.42E^{-06}$; 17.39%), lectin induced complement pathway ($p = 4.42E^{-06}$; 17.39%) and alternative complement pathway ($p = 4.31E^{-04}$; 13.04%) were identified in Biocarta category (Table S6.2; Fig. S6).

4. Discussion

Leptospirosis has been categorized as a globally important infectious disease due to the frequent occurrence of leptospiral infections in both developing and developed countries, specifically the large outbreaks in tropical and subtropical regions leading to considerable adverse effects on human health and economy [3,22]. Incidence of this acute bacterial infection have been found to be high (annual incidence per 100,000 > 10) in quite a few countries of Asia Pacific region including India, Indonesia, Bangladesh, Sri Lanka, Thailand etc. [23]. India being one of the most flood-stricken countries in the South Asian region due to its geographical location and climate, leptospirosis remains as an important endemic environmental infectious disease in this country with multiple devastating outbreaks such as in Orissa (1999), Mumbai (2005) and several parts of the country [23].

Serum and plasma are attractive biological fluids that contain diversity of proteins released by diseased tissue, and serum/plasma proteomics has gained considerable interest for

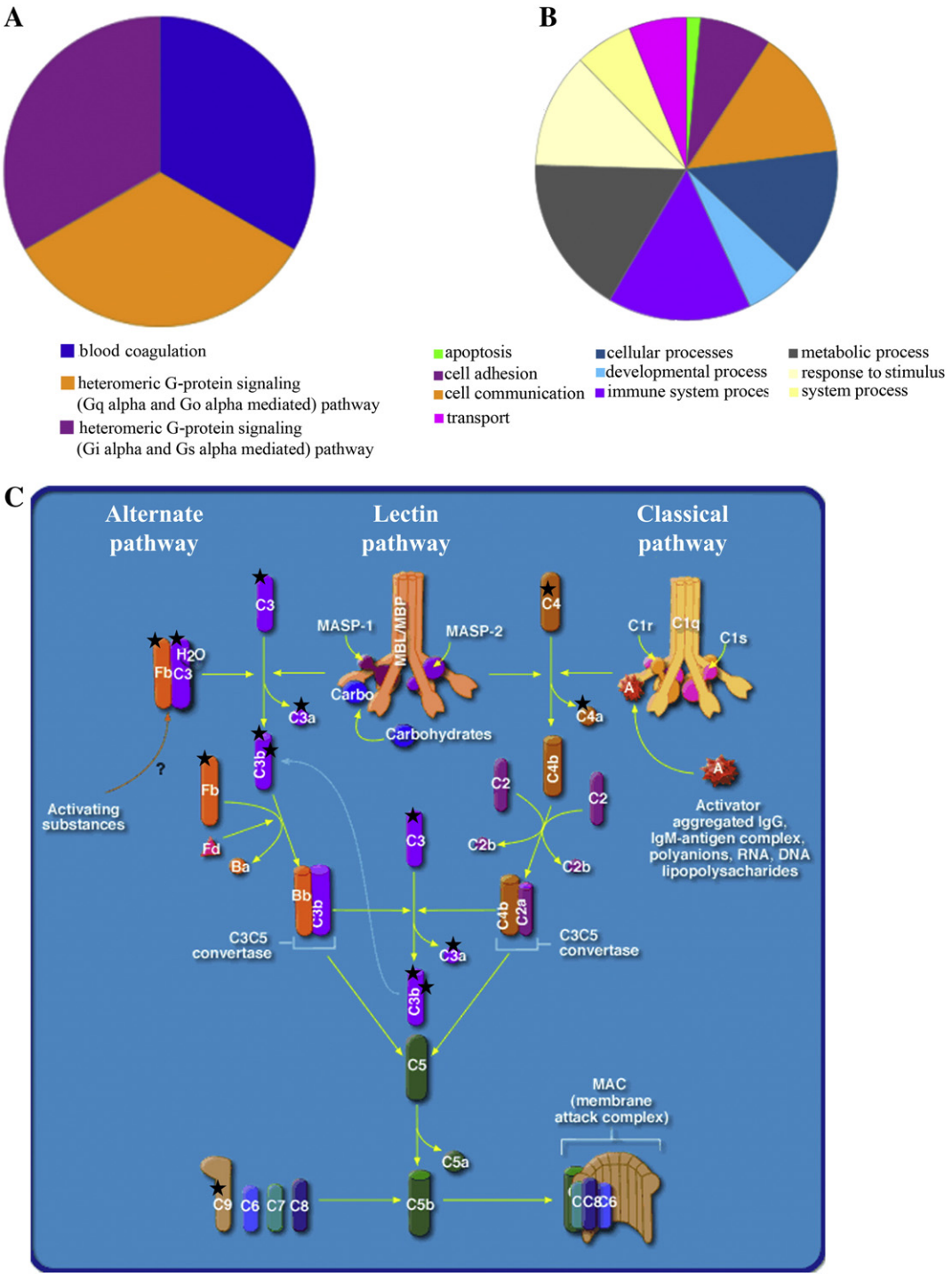

Fig. 4 – Physiological pathways and GO biological process associated with the differentially expressed proteins identified in leptospirosis patients. Pie chart represents (A) pathways (B) biological process obtained in PANTHER analysis. (C) Complement pathways (classical complement pathway, lectin induced complement pathway and alternative complement pathway) identified in DAVID analysis (Biocarta-Pathway Category). Proteins identified in our study are highlighted with black star marks.

the clinical studies, particularly in disease biomarker discovery [10]. Both serum and plasma are components of blood; the liquid portion of blood is referred to as plasma, while removal of the fibrinogen and other clotting factors from plasma results in serum. Applications of serum and plasma as biological fluid for clinical research have their own advantages and limitations. Serum does not contain fibrinogen and other blood clotting factors and has a lesser total protein content compared to plasma, which reduces the unnecessary complexity to some extent making serum more ideal for disease diagnostic purposes.

Very recently, Zimmerman et al. have performed a paired comparison of plasma and serum samples prepared from the same subjects and demonstrated negligible differences in the numbers of peptide and protein identifications or in the overall percentages of semi-tryptic peptides or methionine oxidized peptides between these two biological fluids indicating there is no significant difference between heparinized plasma and serum in terms of overall protein diversity except the depletion of fibrinogen in serum [24].

Earlier reports suggest that proteome level analysis of different biological fluids specifically serum/plasma is very effective in studying disease pathogenesis, pathogen-induced alteration in host, and host responses in different vector-borne infectious diseases like malaria [19], dengue [25] and leishmaniasis [26]. However, over the last decade, only few proteomic studies have been reported on this zoonotic infectious disease. Again, most of the previous proteomic studies have focused on the proteome and immunome profile of the pathogen [11,17,18], and very few studies have investigated the effect of leptospiral infection on host proteome. To this end, two recent studies have reported proteomic analysis of leptospires excreted in urine of chronically infected natural reservoir host *Rattus norvegicus* [15,16], but, no proteome level analysis has been reported hitherto to describe alterations of human serum proteins and related biological pathways due to leptospiral infection. Investigation of the pathogen induced alterations in host serum proteome has immense clinical relevance in light of diagnosis and prognosis.

The present proteomic study aimed to perform human serum proteome analysis of leptospirosis patients from an endemic area in India, and comparative analysis with healthy subjects to investigate disease pathobiology and host responses against leptospiral infections. Additionally, another clinically relevant infectious disease; *falciparum* malaria was analyzed to identify the generic febrile responses. Leptospirosis and malaria have overlapping geographical distributions and coexistence of these two pathogenic infections have been reported from different part of the world, particularly in the tropics [27–30]. Although, these two infections have many similar clinical presentations, interestingly, quite a few serum proteins including complement C3 precursor, ceruloplasmin precursor, complement component C9, complement factor H and inter-alpha-trypsin inhibitor heavy chain H4 precursor exhibited altered expression levels in leptospirosis patients, but not in *falciparum* malaria (febrile controls) (Table S5; Fig. S3). Again, few proteins like complement factor B precursor, Ig kappa chain C region, Ig mu chain C region found to exhibit opposite trend of differential expression between leptospirosis and *falciparum* malaria compared to the healthy controls. However, many of the identified targets found to be similarly modulated in leptospirosis and *falciparum* malaria (Table S5).

All of the leptospirosis patients selected for this study were suffering from preliminary infection with 2–4 days of fever and treated with antibiotics and antipyretics prior to the sample collection. The mean age of the leptospirosis patients, selected for this proteomic analysis was 30.5 years (SD=8.31; range of 23–42; median 26.5) (Table S1). Selection of healthy and febrile control populations with comparable age distribution, with an average value of 28.7 years (SD=5.98; range of 21–34; median 26.5) and 30.4 years (SD=9.60; range of 20–45; median 28.5) respectively, allowed maintaining the uniform population profiles for differential protein expression analysis. Since, rigid inclusion criteria were maintained (Table S1.1) during the selection of patients, and subjects with any significant past history of diseases were excluded, minimum variations were observed in serum proteome profile of the patients suffering from leptospiral infection.

Aiming at analysis of host serum proteome alteration due to leptospirosis, we have identified several differentially expressed proteins and modulation of multiple physiological processes and pathways, including inflammation mediated acute phase responses, complement pathways, heterotrimeric G-protein signaling pathway, coagulation cascade and hemostasis in patients suffering from leptospiral infection. Some of our identified proteins such as Ig mu chain C, clusterin precursor, different complement factors and associated pathways like acute phase response signaling, complement and coagulation cascades, even though not through proteomic studies, have been correlated with leptospiral infection earlier, supporting our findings and enhance the confidence in this study. Further, proteomic analysis revealed differential expression of few serum proteins, such as α-1-antitrypsin precursor (2.29 to 1.81-fold), vitronectin precursor (2.26-fold), α-1-antichymotrypsin precursor (1.40 to 1.68-fold), ceruloplasmin precursor (1.19 to 1.59-fold) up-regulated; and regulator of G-protein signaling 7 (1.62-fold), apolipoprotein A-IV (1.91-fold) down-regulated, which have not been reported previously in the context of leptospirosis (Tables 1 and 2).

Altered expression of multiple acute phase proteins (APPs) including ceruloplasmin, α-1-antichymotrypsin, α-1-antitrypsin, apolipoprotein A-I precursor, inter-alpha-trypsin inhibitor heavy chain H4 precursor has been identified in leptospirosis patients (Table 2). APPs are a group of serum proteins that exhibit differential expression during various acute phase responses involved in host-adaptive and host-defense mechanisms including opsonizing activities, stimulate phagocytic killing of the invading pathogens and many other specific actions such as protein transport, antioxidant activity, and inhibition of serum serine proteinases [31]. Inflammation mediated acute phase signaling has been reported previously in various parasitic and viral infectious diseases like malaria [19], dengue [25] and severe acute respiratory syndrome [32]. Cytokines play a pivotal role in stimulating the production of APPs as a part of the host immune response against the infection. Investigation of the precise biological significance of these APPs and their association with leptospiral infection may provide some insight about the disease pathogenesis.

Our proteomic analysis reveals altered expression of few members of the blood coagulation cascade such as alpha-1-antitrypsin precursor, complement factors B and H etc. in the patients suffering from leptospiral infection (Table S6). The thrombocytopenia has been found to be associated with majority of the leptospirosis patients, the precise reasons and pathophysiological mechanisms liable for bleeding in this zoonosis is not clear [33]. Previous studies have demonstrated disseminated intravascular coagulation (DIC) as an important feature of leptospirosis [34]. Inflammation-induced coagulation activation leading to thrombocytopenia is a consistently detectable phenomenon in infectious diseases and reported in the context of *falciparum* malaria [35] and dengue virus infection [36] earlier. Bleeding

tendency in leptospirosis may arise due to an imbalance in the hemostatic equilibrium and toxic effect exerted by the pathogen on bone marrow leads to thrombocytopenia [37,38]. However, the precise role of activation of coagulation system in the pathogenesis of leptospirosis needs to be established.

Another interesting finding is the modulation of complement pathways, alternative and lectin, in this zoonotic infectious disease (Fig. S6). After being activated by innate immunity system of the host, the complement cascade plays an important role in recognition and destruction of invading pathogens. Previous studies have also shown the activation of complement system's lectin pathway through elevated serum levels of mannose-binding lectin, which exhibit sound correlation with the severity of clinical signs of leptospirosis [39]. In order to survive within host cells and escape the defense system, pathogenic microorganisms adapt versatile mechanisms including inhibition of complement cascades [40]. In this proteomic analysis we have identified down-regulation of few complement factors, including complement C3 precursor (2.21-fold), complement C4 precursor (1.89-fold) and some complement regulatory proteins like clusterin (2.16-fold) in the leptospirosis patients (Table 2). In leptospiral infection, soluble complement regulator factor H prevents complement activation at the C3 stage by inhibiting the C3-convertase and inactivating C3b into iC3b [41]. To get rid of clearance and destruction by host complement system virulent pathogens sometimes acquire fluid-phase regulators of complement pathways such as factor H and C4b-binding protein (C4BP) on the cellular surface [42,43]. Previous studies have demonstrated immune evasion of leptospira species by acquisition of human complement regulator factor H, factor H-related protein 1 (FHR-1) and C4BP [41,44,45]. Decreased expression level of complement factors and regulatory proteins might be a consequence of the leptospiral infection and probably through the deposition of complement inhibition molecules on the pathogen cell surface or by some other unknown mechanisms performed by this pathogenic spirochete after invasion. Detailed investigation of functional aspects of complement factors and regulatory proteins might provide interesting insights about the disease pathogenesis and aid in identification of potential therapeutic targets.

Although, leptospiral infection in human generally remains at a subclinical level or results in mild self-limiting systemic illness, but, it may cause severe infection with life-threatening complications like septic shock, multi-organ failure and lethal pulmonary hemorrhages leading to mortality if not diagnosed and treated timely. In the developing countries, lack of awareness of the disease and inaccessibility of appropriate laboratory diagnostic facilities are the major causes behind the rapid transmission and motility associated with this vector-borne infectious disease [46]. Diagnosis of leptospirosis generally performed through the detection of IgM antibodies against the pathogen by rapid screening tests [47]. Microscopic agglutination test (MAT) and ELISA-based immunoassays are the most common approaches for diagnosis of this acute bacterial infection and carried out at different health facilities. Since serological tests become positive only after one week, hematology and urine analysis is also executed to identify different physiological abnormalities such as increased erythrocyte sedimentation rate, breathlessness, bleeding tendencies, proteinuria, hematuria, thrombocytopenia, elevated levels of alkaline phosphatase, serum creatinine phosphokinase, SGOT and SGPT etc. that can be utilized as the indicators of leptospiral infection [48,49]. Misdiagnosis of leptospirosis sometime occurs due to its broad spectrum of symptoms, which may mimic the clinical signs of many related infectious diseases, such as dengue fever, hantavirus infection and malaria [23]. Moreover, mixed infections with malaria or dengue along with leptospirosis also create complications and difficulties for diagnosis process. Establishment of a panel of serological markers that can confirm leptospiral infection with high accuracy and efficiently discriminate it from other related infectious diseases will be extremely precious from the diagnostic points of view. While, the major emphasis of the study was to provide better insight into the underlying molecular mechanisms of the disease pathogenesis and host immune response in leptospirosis, but, one of the possible outcomes of this study could be establishment of early detection surrogates for the disease to meet the need for better diagnostics and effective therapy. Among the identified differentially expressed serum proteins, apolipoprotein A-I, clusterin, α-1B-glycoprotein precursor, vitronectin precursor, and α-1B-glycoprotein precursor could further be investigated as inflammation-related biomarkers of leptospiral infection.

In summary, this is the first study to investigate *Leptospira* induced alterations in human serum proteome. Comprehensive proteomic analysis using classical 2DE and 2D-DIGE in combination with MALDI-TOF/TOF MS revealed differential expression of several serum proteins in leptospirosis patients compared to healthy individuals. Our results suggest that identified proteins are associated with various essential physiological processes and biological functions. Further investigation of functional properties of proteins identified in this study is likely to elucidate better understanding of disease pathobiology and may help to establish candidate surrogate markers proteins for detection of leptospiral infection and discrimination from other related infectious diseases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.04.007.

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