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Proteome and Immunome of Pathogenic *Leptospira* spp. Revealed by 2DE and 2DE-Immunoblotting with Immune Serum

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SUMMARY In this study, proteomes of two pathogenic *Leptospira* spp., namely *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni and *L. borgpetersenii*, serogroup Tarassovi, serovar Tarassovi, were revealed by using two dimensional gel electrophoresis (2DE)-based-proteomics. Bacterial cells were disrupted in a lysis buffer containing 30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS, 2% IPG buffer pH 3-10 and protease inhibitors and then subjected to sonication in order to solubilize as much as possible the bacterial proteins. The 2DE-separated components of both *Leptospira* homogenates were blotted individually onto membranes and antigenic components (immunomes) were revealed by probing the blots with immune serum of a mouse readily immunized with the homogenate of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni. The immunogenic proteins of the two pathogenic *Leptospira* spp. could be grouped into 10 groups. These are: 1) proteins involved in the bacterial transcription and translation including beta subunit transcription anti-termination protein of DNA polymerase III, elongation factors Tu and Ts, and tRNA (guanine-N1)-methyltransferase; 2) proteins functioning as enzymes for metabolisms and nutrient acquisition including acetyl-Co-A acetyltransferase, putative glutamine synthetase, glyceraldehyde-3-phospahte dehydrogenase, NifU-like protein, 3-oxoacyl-(acyl-carrier-protein) reductase, oxidoreductase, sphingomyelinase C precursor, spermidine synthase, beta subunit of succinyl-CoA synthetase, and succinate dehydrogenase iron-sulfur subunit; 3) proteins/enzymes necessary for energy and electron transfer, *i.e*. electron transfer flavoprotein, and proton-translocating transhydrogenase; 4) enzymes for degradation of misfolded proteins, *i.e.* ATP–dependent Clp protease; 5) molecular chaperone, *i.e.* 60 kDa chaperonin; 6) signal transduction system, *i.e.* response regulator; 7) protein involved in immune evasion in host, *i.e.* peroxiredoxin; 8) cell structure proteins including MreB (cytoskeletal) and flagellin/ periplasmic flagellin; 9) lipoproteins/outer membrane proteins: LipL32, LipL41, LipL45 and OmpL1; and 10) various hypothetical proteins. Many immunogenic proteins are common to both *Leptospira* spp. These proteins not only are the diagnostic targets but also have potential as candidates of a broad spectrum leptospirosis vaccine especially the surface exposed components which should be vulnerable to the host immune effector factors.

Leptospirosis is considered an emerging zoonosis not only in the developing rural tropics but also in the urban areas of the developed, temperate countries.[1-3] The incidence of the disease among humans has been increasing during the past two decades and the clinical manifestations are severe with exceptionally high mortality.[4-6] Currently, leptospirosis vaccine is available only for some veterinary use such as for dogs, cattle and pigs.[7-9] Although the human leptospirosis vaccine was produced in China

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since 1978 and in Cuba in 1998, nevertheless, no human vaccines are licensed for use in other countries.[10-12] Most of the vaccines were prepared from inactivated *Leptospira* spp. whole cells (Bacterin)[12] or outer membrane components[11] which, not only the supply of the vaccines is inadequate owing to the slow growth of the *Leptospira* spp. *in vitro*, but also the vaccines elicited principally antibody to the bacterial lipopolysaccharide (LPS) which conferred limited immunity only to the homologous infection. A broad spectrum leptospirosis vaccine made up of common pathogenic *Leptospira* proteins (native *versus* recombinant) or their coding DNA in an appropriate formulation might protects across serogroups and serovars.

In this study, proteomes and immunomes of two pathogenic *Leptospira* spp. belonging to different genomospecies grown *in vitro* were studied. Common immunogenic proteins among them were identified. These proteins have potential as broad spectrum vaccine candidates.

MATERIALS AND METHODS

Leptospira spp. and mouse immunogen

L. interrogans, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae was grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, Detroit, MI, USA) at 30°C under aerobic conditions. Whole cell *Leptospira* homogenate (mouse immunogen) was prepared. Briefly, the bacteria were collected from the log phase culture by centrifugation at 12,000 x *g*, at 25°C for 30 minutes. The cell pellet was washed three times with 0.15 M phosphate buffered saline, pH 7.4 (PBS) and finally resuspended in a small volume of sterile ultrapure distilled water containing protease inhibitors (Roche Diagnostics GmBH, Germany). The preparation was sonicated at 20 kHz (Model VC750, Bibra Cell™ Sonics & Materials Inc., USA) in an ice bath at 30% amplitude, 2 seconds pulse-on, 2.5 seconds pulse-off, for a total of 5 minutes and the protein content of the homogenate was determined.[13]

Preparation of mouse immune serum to *Leptospira* spp.

A male BALB/c mouse (six weeks old) was immunized intraperitoneally (i.p.) with 50 μg whole cell homogenate of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae (100 μl) mixed with an equal volume of complete Freund's adjuvant. Two booster doses were given at two-week intervals using the same dose of the immunogen emulsified in incomplete Freund's adjuvant and the same route. Fourteen days after the second i.p. booster, the mouse was given an intravenously injection with ~1 x 10^6 cells of heat killed *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae. The mouse was bled three days after the intravenous injection, the immune serum was collected and the antibody titer was determined by indirect ELISA against the homologous antigen as previously described.[14]

Two dimensional gel electrophoresis (2DE)

L. interrogans, serogroup Icterohemorrhagiae, serovar Copenhageni and *L. borgpetersenii,* serogroup Tarassovi, serovar Tarassovi were separately grown in liquid EMJH medium. Bacterial cells were collected from 3-4 week culture by centrifugation as above. The cells were washed three times with a wash buffer containing 10 mM Tris and 5 mM magnesium acetate. Bacterial cells in the pellet of the last wash were resuspended in a lysis buffer (30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS, 2% IPG buffer pH 3-10) containing the protease inhibitors. The preparation was kept on ice for 10 minutes and subjected to sonication as above. After sonication, the preparation was centrifuged to remove the cell debris. The supernatant was transferred to a new tube and the pH was adjusted to 8.5 by careful adding with 50 mM sodium hydroxide. The *Leptospira* homogenate was treated with a 2D-Clean-up reagent (Amersham Biosciences, CA, USA) to eliminate detergents, salts, lipids, phenolics, and nucleic acids. After cleaning, each preparation was dissolved in a DeStreak Rehydration solution (Amersham Biosciences). The protein content was determined using the 2D-Quant kit (PlusOne, Amersham Biosciences) before the first dimension electrophoresis. The *Leptospira* homogenate (30-60 μg protein) was added to the DeStreak Rehydration solution containing 0.18 M dithiothreitol (DTT) and 0.5% non-linear (NL) pH 3-10 IPG buffer (Amersham Biosciences). Each preparation (125 μl) was loaded on a 7 cm rehydrated-IPG strip of pH 3-10. Care was taken not to produce any bubbles. The IPG strip was placed (right

side down) into the strip holder containing the sample, then dry strip cover fluid was added to the strip holder. The strip holder was placed into the Ettan IPG Phor Electrofocusing System (Amersham Biosciences) and the IPG strip was allowed to rehydrate at 20°C for 12 hours. Electrophoresis was performed at 20°C using the following parameters: 300 Volts until 200 Volt-hours (Vh) were obtained; 1,000 Volts until 300 Vh were obtained; a gradient step at 5,000 Volts until 4,500 Vh were obtained; and step and hold at 5,000 Volts until 3,000 Vh were obtained. For the second dimension gel electrophoresis, the electrofocused IPG strip was equilibrated in 10 ml SDS-equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% Bromphenol blue) containing 100 mg DTT for 15 minutes. It was placed in 10 ml of the equilibration buffer containing 250 mg iodoacetamide for 15 minutes. The strip was washed with electrode buffer and overlaid onto a 12.5% polyacrylamide gel (8 x 9.5 cm) cast in SE260 (Mini Vertical, Amersham Biosciences). SDS-PAGE was carried out at 10 mAmp/gel during the first 15 minutes and at 20 mAmp/gel until the tracking dye reached the lower edge of the gel. After SDS-PAGE, the gel was either stained with Colloidal Commassie Brilliant Blue G-250 (CBB) dye or the separated components in the gel were electro-transblotted onto a PVDF membrane for further 2DE-immunoblotting with mouse immune serum to identify all *Leptospira* antigenic components (immunome).

2DE-immunoblotting

The PVDF membrane blotted with the 2DE-separated *Leptospira* components was immersed in a blocking solution [3% BSA in Tris buffered saline pH 7.6 (TBS)] at 25°C for 1 hour. After thorough washing with a wash buffer [TBS containing 0.05% Tween-20 (TBS-T)], the blot was incubated with mouse immune serum at 25°C overnight. After the excess serum was discarded and the membrane was thoroughly washed with the wash buffer, it was placed in a solution of goat anti-mouse IgG-alkaline phosphatase conjugate (Southern Biotech, AL, USA) diluted 1:2,000 in TBS-T at 25°C for 1 hour. The membrane was washed with TBS-T and then placed in 0.15 M Tris-HCl, pH 9.6, for 10 minutes before incubating with BCIP/NBT substrate (KPL, Gaithersburg, MD, USA) in the dark for 10 minutes. The enzyme-substrate reaction was stopped by rinsing the membrane with de-ionized water and air-dried.

Liquid chromatography/tandem mass spectrometry (LC/MS-MS)

Gel plugs containing proteins corresponding to the spots reacting to the mouse immune serum in the 2DE-immunoblots were carefully excised from the CBB-stained-2DE-gel. The gel plugs were destained using a wash solution containing 50% methanol and 5% acetic acid in UDW at 25°C overnight. Individual gel plugs were rinsed a few times in a period of 2-3 hours with fresh aliquots of the wash solution. Proteins in the gel plugs were digested with trypsin.[15] For peptide analysis, the LC/MS-MS model Finnigan LTQ Linear Ion Trap Mass Spectrometer was used. The HPLC system was a Finnigan Surveyor™ MS pump with a flow splitter. The column size was 0.18 x 100 mm, *i.e.* C18 (Thermo Electron Corporation). The flow rate was 200 µl per minute. The two mobile phases were: A) 0.1% formic acid in water, and B) 0.1% formic acid in acetonitrile. After washing to remove unbound peptides with A, the following gradients of B were applied to the column: 0-60% B in A for 20 min, 65-80% B for 5 minutes, and 80-0% B for 2 minutes. Peptides in the eluates were analyzed by mass spectrometry (Finnigan LTQ) which used NanoSpray, positive ion in ionization mode at a capillary temperature of 200°C with a 1.8 kV spray needle. The scan sequence was full-scan MS and MS-MS scan with a mass range of 400-1600 *m*/*z*. The acquisition modes were Normal, Data Dependent™ and Dynamic Exclusion™.

Database search

The ion spectra of peptides generated by the mass spectrometry were interpreted using the Turbo SEQUEST algorithm in the BioWorks™ 3.1SR1 software package (Thermo Electron) and nr.fasta database. Protein search parameter included a precursor peptide mass tolerance of ± 1.25 amu, fragment mass tolerance of ± 0.4 amu, methionine (M) oxidation, and threonine (T) or serine (S) phosphorylation. For the tryptic status requirement, at least one end of the peptide had to be a tryptic site. The identified peptides were further evaluated using charge state *versus* cross-correlation number (X_{corr}). The criteria for positive identification of peptides were X_{corr} >1.5 for sin-

gly charged ions, X_{corr} >2.0 for doubly charged ions, and X_{corr} > 2.5 for triply charged ions. A delta correlation of (ΔC_n) >0.8 was used as cut-off for peptide acceptance. The minimum number of one peptide per protein was specified by the software.

RESULTS

The BALB/c mouse immunized with the homogenate of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae, had the indirect ELISA titer of 1:64,000 against the homologous antigen.

Proteome of whole cell homogenate of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni after 2DE at NL pH 3-10 isoelectric focusing, SDS-PAGE and CBB staining is shown in Fig. 1A. Antigenic components in the homogenates of this *Leptospira* spp. reacted to antibodies in the mouse immune serum, *i.e.* immunome, are shown in Fig. 1B. Thirteen gel plugs containing *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni proteins cut from CBB stained gel , *i.e.* circles no. 1-13 of Fig. 1A correspond to the antigenic components (immunome) shown in circles no. 1-13 of Fig. 1B, respectively, were subjected to LC/MS-MS to generate the tryptic digested peptide mass maps. Orthologous protein sequences of the database matched with peptides fragments generated from the proteins in gel plugs no. 1-13 were identified (Table 1).

Proteome of homogenate of *L. borgpeterseii*, serogroup Tarassovi, serovar Tarassovi after 2DE at NL pH 3-10 isoelectric focusing, SDS-PAGE and CBB staining are shown in Fig. 2A. Antigenic components in the homogenate of this *Leptospira* spp. reacted to antibodies in the mouse immune serum, *i.e.* immunome, are shown in Fig. 2B. Gel plugs containing proteins in circles no. 1-19 of the CBB stained gel in Fig. 2A which matched with the antigenic components in circles no. 1-19 of Fig. 2B, respectively, were cut from the CBB stained gel of Fig. 2A and subjected to LC/MS-MS for generating tryptic digested peptide mass fingerprints. Table 2 lists orthologous proteins of the database matched with the peptide fragments generated from the proteins in circles no. 1-22 of Fig. 2A after BLAST search.

The antigenic proteins common to the two pathogenic *Leptospira* spp. are summarized and listed in Table 3.

DISCUSSION

In this study, the proteomes of two pathogenic *Leptospira* spp. were studied using the 2DE-based method. The 2DE-based proteomics has some limitations, *i.e.,* the 2DE is not suitable for studying components in highly complex protein mixtures or membrane proteins of low solubility[16,17] and currently available IPG strips for the first dimensional protein separation cannot be used for extremely acidic or basic proteins with a pI lower than 3 or higher than 12, respectively.[16-18] Nevertheless, the *Leptospira* components were readily revealed by the proteomic conditions used in this study. In order to solubilize as much as possible the *Leptospira* spp. proteins especially the outer membrane lipoproteins we have used the lysis buffer containing both thiourea and urea at high molarity, *i.e.* 2 M and 7 M, respectively. The buffer was found to solubilize most if not all of the bacterial proteins as there were only minute amounts of the sediments after high speed centrifugation of the *Leptospira* spp. homogenates in the lysis buffer. Moreover, the IPG buffer and IPG strip of non-linear pH 3-10 were found to be suitable for the first dimensional separation of the bacterial homogenates as most proteins were focused within the pH range used and almost nil were located at the extremely low or high pH (Fig. 1 and 2). The protein components could be well separated and individual spots were clearly seen after the colloidal CBB staining. Thus, there is no need for the IPG strip of more extreme pH and the narrower pI range for the separation.

The immune mouse serum had satisfactorily high titer after immunization with the *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae using the indicated routes, doses and schedule. The antibodies in the immune serum could recognize many immunogenic proteins in the homogenates of the heterologous species, serogroups and serovars, *i.e. L. interrogans,* serogroup Icterohaemorrhagiae serovar Copenhageni and heterologous serogroup and serovar, *i.e. L. borpetersenni*, serogroup Tarassovi, serovar Tarassovi. Unfortunately, the immunome of the *L. interrogans*, sero-

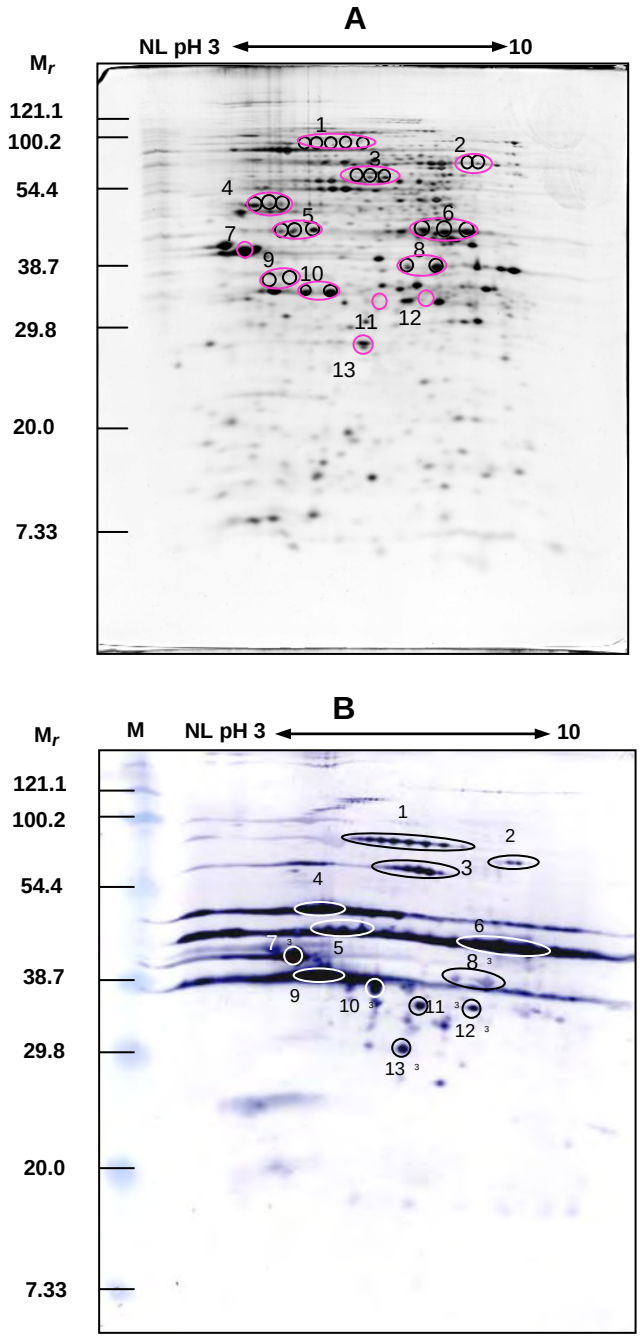

Fig. 1 Proteome and immunome of whole cell homogenate of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni. A, The proteome revealed by 2DE at NL pH 3-10 isoelectric focusing, SDS-PAGE and CBB staining. B, Protein components in the homogenate that reacted with the antibodies in the mouse immune serum (immunome) as revealed by 2DE-immunoblotting. Circles and numbers (1-13) in A are proteins that were cut from the CBB stained gel and subjected to LC/MS-MS to generate peptide mass maps and ortholog search. They are respectively corresponded to those proteins in circles and numbers in B.

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni separated by 2DE at non-linear pH 3-10

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
1	NP_712836	60 kDa chaperonin [*Leptospira interrogans* serovar Lai str. 56601] K.AQEAVGSLK.L K.AQEAVGSLK.L K.AVTAAVESIQK.R K.FGAPTITK.D	6.0	5.15	70,556.9	57,969.4	-	4	30.19
2	NP_711210	Sphingomyelinase C precursor (*L. interrogans* serovar Lai str. 56601) K.DYDTYSR.N K.DYDTYSR.N R.ANQFDDIR.N K.KYDQVSFQSAANGK.Y K.ESNEYYDM*ISR.L K.YDQVSFQSAANGK.Y R.ANQFDDIR.N K.YDQVSFQSAANGK.Y R.NFIYSK.N R.EEYPYQTDVVGR.T K.ADAVIETDFTK.F K.ADAVIETDFTK.F R.SYSLVNGGVVILSK.W	7.6	5.92	60,476.9	71,029.6	13.30	13	86.26
	YP_003378	Hypothetical protein LIC13477 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.AYLDTTQGK.I R.NQAAVDTEIAER.L R.VVFTPGTGVTVNK.D	7.6		60,476.9	59,408.2	6.50	3	30.17
3	YP_002712	Acetyl-CoA acetyltransferase (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.GVHPNNM*GIGQAVATK.A K.GVHPNNM*GIGQAVATK.A R.EGLVENPTR.M R.AM*LM*FDNPGM*K.F R.VIANLVGM*R.D K.PLAICTPR.R R.TPFAQIAK.A R.SDGAAGVIVTTVEK.A R.SDGAAGVIVTTVEK.A K.YSPYIIPM*K.D K.ALLDETGIK.I K.ALLDETGIK.I R.LIANAIM*DLK.E K.M*DTGWDWEK.S K.LAFESFK.R K.LLPDNLPEGVELR.D K.LLPDNLPEGVELR.D	5.9	5.97	57,326.9	47,280.1	29.93	17	126.19
	YP_002339	Putative glutamine synthetase protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.GIQQM*PHTLR.E K.SCAAFTNASTNSYK.R K.SCAAFTNASTNSYK.R R.TPSEVIAYAK.A R.TPSEVIAYAK.A R.TPSEVIAYAK.A R.IPFVNGDK.A R.IPFVNGDK.A K.FGTLIEAADNVQK.L K.FGTLIEAADNVQK.L K.NGVNLFAGK.G K.NGVNLFAGK.G	5.9	5.89	57,326.9	53,106.7	13.53	12	58.24

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni, separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
	YP_002404	Dihydrolipoamide succinyltransferase (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.VKEAIEDPSR.L K.VKEAIEDPSR.L K.NGQITKEDVLK.A K.ATSSVSNASVNVGTPAAVK.A K.ATSSVSNASVNVGTPAAVK.A K.ATSSVSNASVNVGTPAAVK.A K.NYYDIGVAVGGPK.G K.ATLTLPEIPK.A	5.9	6.77	57,326.9	45,197.8	15.00	8	50.24
4	YP_002497	Succinyl-CoA synthetase beta subunit (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) R.HADNAAFR.D K.TKEDAIAAVDK.I K.QNEIIAGDCK.I K.TKEDAIAAVDK.I K.TGGSVVVVK.A K.QNEIIAGDCK.I K.TGGSVVVVK.A K.IILGDPNVK.G K.IILGDPNVK.G R.CDM*VAEGIIEAAK.A K.VYLEQGIDIAK.E K.IDLDENALYR.H K.VYLEQGIDIAK.E K.IAIDPGIGLQVNQAR.Q K.ANVPFGVVIDK.K K.ANVPFGVVIDK.K	5.5	5.59	45,986.4	41,890.1	27.44	16	98.22
	BAE48275	Lipoprotein LipL41 (*L. interrogans* serovar Icterohaemorrhagiae) K.DVNTGNEPVSK.P K.DVNTGNEPVSKPTGVR.M R.VKATFAVDESNAK.- R.NVGLAVEAPK.K R.NVGLAVEAPK.K K.VFEAFDK.E K.VFEAFDK.E R.ADAINEASLSLTGITK.N R.ADAINEASLSLTGITK.N R.M*M*LIPLDATLIK.V R.M*M*LIPLDATLIK.V	5.5	8.59	45,986.4	38,936.6	20.84	11	68.26
	YP_000046	Proton-translocating transhydrogenase, subunit alpha part 1 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.HGQNVVTPK.G K.HGQNVVTPK.G K.NLAGSIPADASK.M K.NLAGSIPADASK.M R.AQSM*DVLSSQATVAGYK.A R.AQSMDVLSSQATVAGYK.A R.AQSM*DVLSSQATVAGYK.A R.AQSMDVLSSQATVAGYK.A R.VSVTPDIVDALK.K K.ADVIITTALIPGK.K	5.5	9.90	45,986.4	41,418.6	16.19	10	58.29

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
4 (con.)	AAN64010	DNA polymerase III, beta subunit (*L. interrogans*) K.TSYAIAHEDQR.F K.ISGM*DADEIK.T R.QVLTAAEEPSR.Q R.QVLTAAEEPSR.Q K.TSVPADVTQEGNVSLPAK.Q K.TSVPADVTQEGNVSLPAK.Q K.LIFVGTDGR.R	5.5	4.89	45,986.4	41,424.9	15.82	7	46.17
	YP_002791	Elongation factor Tu (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.ADM*LAADER.A K.ADM*LAADER.A K.AVAYDQIDNAPEEK.A K.ALEGDESEIGM*PAILK.L K.ALEGDESEIGM*PAILK.L	5.5	5.69	45,986.4	43,573.8	9.73	5	30.21
5	YP_001227	MreB (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.YIKPGIR.- R.TLELESNEIRK.A R.TPGEIVAIRPM*K.D R.TPGEIVAIRPM*K.D R.NQYNLVVGER.T K.LTIGNAFPEKK.A R.TLELESNEIR.K R.TPGEIVAIR.P K.ETGVPVFR.A K.LTIGNAFPEK.K R.TPPELASDIVER.G R.TGGDEFDEAIIK.Y R.TPPELASDIVER.G R.TPPELASDIVER.G R.TGGDEFDEAIIK.Y R.TGGDEFDEAIIK.Y R.TGGDEFDEAIIK.Y R.TGGDEFDEAIIK.Y R.IVIGVPSGITEVER.R R.GIVLTGGGCLLR.G R.AENPLTCVVLGTGK.Y R.AENPLTCVVLGTGK.Y K.DGVIADFETVEK.M K.DGVIADFETVEK.M	5.5	5.21	42,061.0	36,835.4	39.71	24	148.23
	YP_000467	Hypothetical protein LIC10483 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.FDHDGVDK.L K.TKTQPDFQK.Y K.SFVNTVVGTQHK.F R.AFGNYNIK.V K.TGNIPVLEK.L K.TGNIPVLEK.L K.YEGQPGEIVWK.F K.DILNSI.- R.LSDFISEVVLK.N R.LSDFISEVVLK.N R.LSDFISEVVLK.N	5.5	5.40	42,061.0	36,148.2	22.42	11	80.22
	YP_002024	Glyceraldehyde-3-phosphate dehydrogenase (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) R.FQGTVEHTEK.E R.GAM*QNIIPASTGAAK.A K.DIPTFVM*GVNNEK.Y	5.5	7.70	42,061.0	36,647.9	8.41	3	28.18

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
5 (con.)	YP_003074	Hypothetical protein LIC13166 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.NGGASEDQM*FK.Y K.NGGASEDQM*FK.Y R.LALSNYQSGVNTILK.M		8.76		36,212.1	8.50	3	20.21
6	YP_003074	Hypothetical protein LIC13166 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.YGKDSPEYK.H K.NGGASEDQM*FK.Y K.NGGASEDQM*FK.Y K.DEVISVRP K.DEVISVRP.- R.LALSNYQSGVNTILK.M R.LALSNYQSGVNTILK.M	8.1	8.78	41,032.8	36,212.1	14.10	7	50.21
	YP_002198	Hypothetical protein LIC12263 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.VEAGNTTTEVGDK.V R.ISM*M*DM*SNEEIK.T R.ISM*M*DM*SNEEIK.T	81		41,032.8	37,517.8	7.50	7	20.18
7	YP_001839	Flagellin protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.FQNNEVAK.N K.NM*ETLSSGM*R.I R.VYIATM*TAK.S K.NM*ETLSSGMR.I K.NMETLSSGMR.I K.NMETLSSGMR.I R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N R.DTDM*AEETVAFTK.N K.GLM*VAYENIQASESR.I K.GLM*VAYENIQASESR.I R.VLAIQSSNGIYSAEDR.Q R.VLAIQSSNGIYSAEDR.Q R.ANLGAYFNR.L K.GLM*VAYENIQASESR.I K.GLMVAYENIQASESR.I K.GLMVAYENIQASESR.I K.M*ALLQGDFAR.G R.VLAIQSSNGIYSAEDR.Q K.MALLQGDFAR.G K.MALLQGDFAR.G K.GLM*VAYENIQASESR.I K.GLMVAYENIQASESR.I K.GLM*VAYENIQASESR.I K.GLMVAYENIQASESR.I R.IRDTDM*AEETVAFTK.N R.ANLGAYFNR.L R.PQSVLQLLR.- R.ANLGAYFNR.L K.M*ALLQGDFAR.G R.VLAIQSSNGIYSAEDR.Q R.VLAIQSSNGIYSAEDR.Q K.GLM*VAYENIQASESR.I K.GLMVAYENIQASESR.I K.GLM*VAYENIQASESR.I K.M*ALLQGDFAR.G R.ANLGAYFNR.L	5.2	6.66	38,850.6	31,305.3	35.94	58	138.29

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
7 (con.)		R.VLAIQSSNGIYSAEDR.Q R.IRDTDM*AEETVAFTK.N K.GLM*VAYENIQASESR.I K.GLMVAYENIQASESR.I K.GLM*VAYENIQASESR.I K.GLMVAYENIQASESR.I R.IRDTDM*AEETVAFTK.N K.M*ALLQGDFAR.G R.ANLGAYFNR.L R.VLAIQSSNGIYSAEDR.Q K.M*ALLQGDFAR.G R.ANLGAYFNR.L R.VLAIQSSNGIYSAEDR.Q R.VLAIQSSNGIYSAEDR.Q R.ANLGAYFNR.L R.ANLGAYFNR.L K.M*ALLQGDFAR.G R.ANLGAYFNR.L R.ANLGAYFNR.L							
	NP_712200	Periplasmic flagellin (*L. interrogans* serovar Lai str. 56601) R.ADM*GAYYNR.L R.ADM*GAYYNR.L R.IASQAEFNK.F R.IASQAEFNK.F K.GLM*GAYENM*QASESR.I K.GLM*GAYENM*QASESR.I R.INSAADDASGLAVSEK.L R.INSAADDASGLAVSEK.L K.FNELAVDK.T K.LFEGQFAR.G	5.2	9.24	38,850.6	31,271.5	22.97	10	60.26
	YP_000467	Hypothetical protein LIC10483 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.FDHDGVDK.L K.SLLDGGLITQEEFDTK.K R.LSDFISEVVLK.N	5.2		38,850.6	36,148.2		3	30.27
8	YP_001839	Flagellin protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.FQNNEVAK.N K.NM*ETLSSGM*R.I K.NM*ETLSSGMR.I K.NMETLSSGMR.I K.NMETLSSGM*R.I R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N -.M*IINHNLAAINSHR.V R.VLAIQSSNGIYSAEDR.Q K.M*ALLQGDFAR.G K.M*ALLQGDFAR.G R.ANLGAYFNR.L R.ANLGAYFNR.L K.FQNNEVAK.N	7	6.66	35,684.2	31,305.3	29.18	14	92.25
9	YP_001839	Flagellin protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.NM*ETLSSGM*R.I R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N R.IRDTDM*AEETVAFTK.N	5.4	6.66	35,684.2	31,305.3	26.33	11	60.22

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni, separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
9 (con.)		-.M*IINHNLAAINSHR.V R.VLAIQSSNGIYSAEDR.Q R.VLAIQSSNGIYSAEDR.Q K.M*ALLQGDFAR.G R.ANLGAYFNR.L R.ANLGAYFNR.L K.M*ALLQGDFAR.G							
	YP_001601	LipL45 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.KLESEVAAR.Q K.AAVLEAERK.K R.TIVTVDKDTTEK.M R.TIVTVDKDTTEK.M K.IQQTVASSEIVLEK.N	5.4	7.37	35,684.2	42,289.1	11.28	5	40.20
	YP_001344	Sigma factor WhiG (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.YNQQDETELWK.S K.YNQQDETELWK.S K.EIGEVLEVTESR.I K.QLEQIIGM*LENK.E K.QLEQIIGM*LENK.E R.GSIFDELR.S	5.4	5.09	35,684.2	30,871.2	16.17	6	40.19
10	YP_000350	Electron transport flavoprotein beta subunit (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) R.TAYAM*GADR.A R.TAYAM*GADR.A K.SAADLGSPASK.I K.SAADLGSPASK.I K.SAADLGSPASK.I K.SAADLGSPASK.I R.TAYAMGADR.A K.EVEGGTQTVETSTPVALTAQK.G K.AENADVIIGGR.Q K.AENADVIIGGR.Q K.AENADVIIGGR.Q K.EVEGGTQTVETSTPVALTAQK.G K.EVEGGTQTVETSTPVALTAQK.G K.EVEGGTQTVETSTPVALTAQK.G K.AENADVIIGGR.Q K.AENADVIIGGR.Q K.AENADVIIGGR.Q K.AENADVIIGGR.Q	5.7	5.47	35,214.7	27,067.8	20.95	18	50.29
	YP_003032	Hypothetical protein LIC13123 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.SLENHPYLK.F R.TSM*ALGEVDLK.A R.TSM*ALGEVDLK.A R.AYFEQIR.K K.ETVNFLVSTLEK.E K.ETVNFLVSTLEK.E	5.7	6.44	35,214.7	33,139.0	13.40	6	32.17
	NP_710593	Electron transfer flavoprotein beta-subunit *(L. interrogans* serovar Lai str. 56601) R.KLEAADANAFASQLVK.A R.KLEAADANAFASQLVK.A R.KLEAADANAFASQLVK.A R.KLEAADANAFASQLVK.A K.LEAADANAFASQLVK.A K.LEAADANAFASQLVK.A K.LEAADANAFASQLVK.A	5.7	5.47	35,214.7	27,083.84	6.35	7	20.3

Table 1 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 13 of homogenate of *L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni, separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
	YP_003638	response regulator [Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130] K.AGIKPNVGASGEIIR.G K.AGIKPNVGASGEIIR.G K.AGIKPNVGASGEIIR.G R.NDENTPIFIVTAR.N R.NDENTPIFIVTAR.N	5.7	5.48	35,214.7		11.52	5	20.24
11	YP_001595	hypothetical protein LIC11637 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) R.AESGAM*VAM*SPTVK.M R.AESGAM*VAM*SPTVK.M R.AESGAMVAM*SPTVK.M R.AESGAM*VAMSPTVK.M R.AESGAMVAM*SPTVK.M R.AESGAM*VAMSPTVK.M K.AEGGVFASAK.R K.AEGGVFASAK.R R.GAYVAGSESITIDSK.W K.MNGEDLILSR.G K.MNGEDLILSR.G	6.0	5.69	33,071.0	24,343.3	21.7	11	58.23
	YP_000350	Electron transport flavoprotein beta subunit (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) R.TAYAM*GADR.A K.SAADLGSPASK.I K.AENADVIIGGR.Q K.EVEGGTQTVETSTPVALTAQK.G K.EVEGGTQTVETSTPVALTAQK.G K.EVEGGTQTVETSTPVALTAQK.G	6.0	5.47	33,071.0	27,067.8	20.6	6	40.27
	YP_001839	flagellin protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) R.IRDTDM*AEETVAFTK.N K.M*ALLQGDFAR.G R.ANLGAYFNR.L R.ANLGAYFNR.L	6.0	6.66	33,071.0	27,067.8	12.1	4	30.2
12	YP_000627	hypothetical protein LIC10643 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.LSNSSLETK.S K.GNLIQYEDR.Y K.DSVQNPLLILNEK.Q K.DSVQNPLLILNEK.Q	6.8		32,678.8		7.16	4	30.19
	BAE48275	lipoprotein LipL41 (*L. interrogans* serovar Icterohaemorrhagiae) K.DVNTGNEPVSK.P K.DVNTGNEPVSKPTGVR.M K.VAGFAASM*ATGK.D K.VAGFAASM*ATGK.D	6.8	8.59	32,678.8	38,936.6	7.89	4	30.17
13	YP_001379	ATP-dependent Clp protease, proteolytic subunit (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) K.RSALPNAR.I K.TVEQIQKDTER.N R.NFYM*TADEAK.N R.NFYM*TADEAK.N R.NFYMTADEAK.N	5.9	5.58	29,588.7	22,112.1	14.65	5	36.17

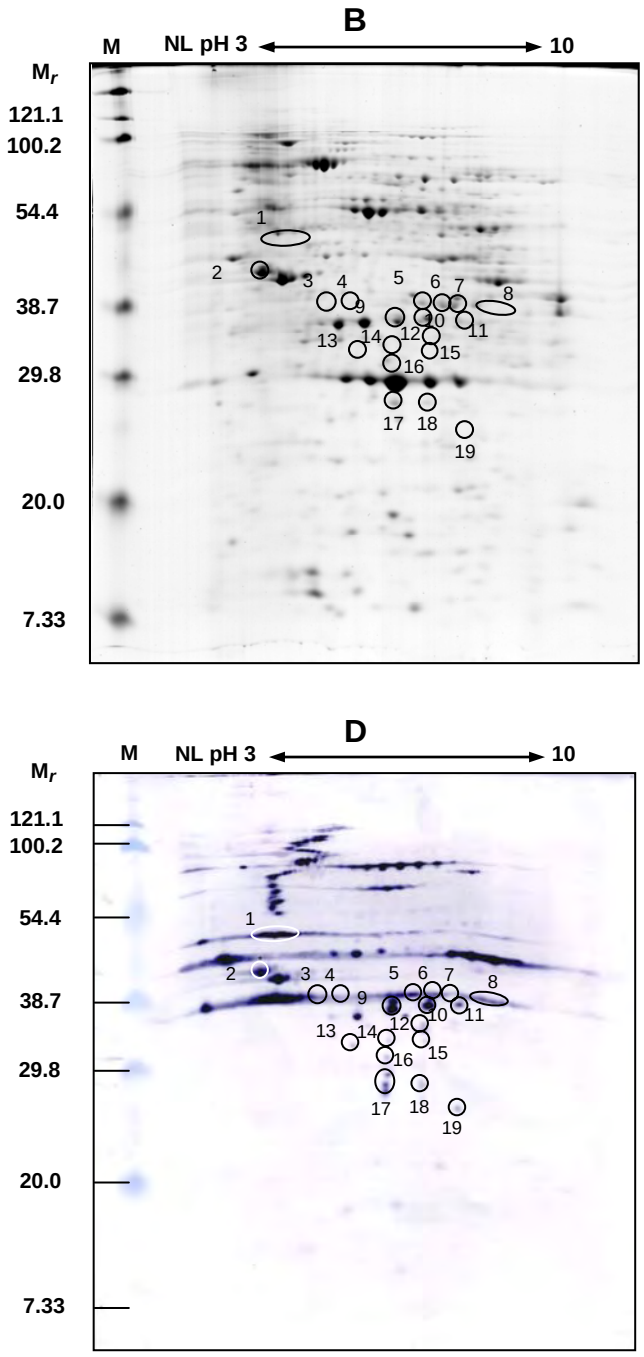

Fig. 2 Proteome and immunome of whole cell homogenatesof and *L. borgpetersenii*, serogroup Tarassovi, serovar Tarassovi. A, Proteome of the homogenate after 2DE at NL pH 3-10 isoelectric focusing, SDS-PAGE and CBB staining. B, Components in the homogenate that reacted to antibodies in the mouse immune serum (immunome) as revealed by 2DE-immunoblotting. Circles and numbers in A are locations of proteins in 19 gel plugs that were cut from the CBB stained gel, subjected to LC/MS-MS and database search for orthologous proteins. Circles and numbers in B are the respectively corresponded proteins to those in A.

Table 2 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 19 of homogenate of *L. borgpertersenii,* serogroup Tarassovi, serovar Tarassovi separated by 2DE at non-linear pH 3-10

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
1	AAS21807	LipL41 (*L. borgpetersenii*) K.EVNTGNEPVSKPTGVR.M K.EVNTGNEPVSKPTGVR.M R.NVGLAVEPPKK.S K.ANLATYYFSVGDFEK.S K.ANLATYYFSVGDFEK.S K.EVNTGNEPVSKPTGVR.M	7.3	5.55	46,400.0	34,113.8	18.65	6	40.30
	BAE48275	Lipoprotein LipL41 (*L. interrogans* serovar Icterohaemorrhagiae) K.VFVKDEDEEVK.E K.VAGFAASM*ATGK.D	7.3	8.59	46,400.0	38,937.6	6.48	2	20.20
2	YP_001839	Flagellin protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.NM*ETLSSGM*R.- -.VLAIQSSNGIYSAEDR.- -.M*ALLQGDFAR.-	4.8	6.54	28,498.4	31,306.3	12.81	3	30.24
3	NP_712836	60 kDa chaperonin (*L. interrogans* serovar Lai str. 56601) -.VTVDKENTTIIEGK.- -.LENTTLQM*LGR.- -.LENTTLQMLGR.- -.SIETTLDVVEGM*QFDR.- -.SMLEDIAILTGGQVISEDLGM*K.- -.SMLEDIAILTGGQVISEDLGM*K.- -.SMLEDIAILTGGQVISEDLGM*K.-	5.3	5.29	36,645.6	57,970.4	11.54	7	64.33
	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) .M*SAIM*PDQIAK. .M*SAIM*PDQIAK. .GSFVASVGLLFPPGIPGVSPLIHSNPEELQK	5.3	5.74	36,645.6	27,494.0	16.73	3	30.21
4	YP_000467	Hypothetical protein LIC10483 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.YGLEVVDFFIQSINFDQNDPNFQK.- -.YGLEVVDFFIQSINFDQNDPNFQK.-	5.4	5.49	35,000.0	36,149.2	7.27	2	20.34
	AAQ98019	Lip L32 outer membrane protein (*L. interrogans* serovar Icterohaemorrhagiae) -.M*SAIM*PDQIAK.- -.ISFTTYKPGEVK.-	5.4	5.79	35,000.0	23,502.6	10.80	2	20.18
5	NP_714855	Spermidine synthase (*L. interrogans* serovar Lai str. 56601) -.RPFYETM*ANCLK.- -.RPFYETM*ANCLK.- -.IDVFESVGFGR.- -.IDVFESVGFGR.- -.GVIDVCYEYFPEIANAM*K.- -.ELFNFIPEIFK.- -.ELFNFIPEIFK.-	5.9	5.89	36,645.6	32,088.9	18.51	7	70.21
	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) -.M*SAIM*PDQIAK.- -.PGQAPDGLVDGNK.- -.PGQAPDGLVDGNK.- -.ISFTTYKPGEVK.- -.M*SAIM*PDQIAK.- -.AYYLYVWIPAVIAEM*GVR.-	5.9	5.74	36,645.6	27,494.1	39.84	6	60.19

Table 2 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 19 of homogenate of *L. borgpertersenii,* serogroup Tarassovi, serovar Tarassovi separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
6	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) -.M*SAIM*PDQIAK.- -.PGQAPDGLVDGNK.- -.SFDDLKNIDTK.- -.SFDDLKNIDTK.- -.M*SAIM*PDQIAK.- -.M*SAIM*PDQIAK.- -.ISFTTYKPGEVK.- -.M*ISPTGEIGEPGDGDLVSDAFK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.-	6.1	5.74	36,645.6	27,494.1	17.13	10	100.28
	AAV88032	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Javanica) -.TFLPYGSVINYYGYVKPGQAPDGLVDGNK.- -.TFLPYGSVINYYGYVK.- -.TFLPYGSVINYYGYVK.- -.TFLPYGSVINYYGYVK.-	6.1	5.37	36,645.6	25,121.4	12.78	4	40.26
7	NP_710593	Electron transfer flavoprotein beta-subunit (*L. interrogans* serovar Lai str. 56601) -.TAYAM*GADR.- -.QVPDTETSIKVGDK.- -.ATKEVEGGTQTVETSTPVALTAQK.- -.EVEGGTQTVETSTPVALTAQK.- -.HGGEVIAVSLGPDR.- -.HGGEVIAVSLGPDR.- -.IEIVGLEPPPPR.- -.IEIVGLEPPPPR.- -.WIISPYDEFAIEEGIR.- -.WIISPYDEFAIEEGIR.- -.HGGEVIAVSLGPDRVVEALR.-	6.3	5.58	36,645.6	27,084.9	37.55	11	110
	AAQ98019	Lip L32 outer membrane protein (*L. interrogans* serovar Icterohaemorrhagiae) -.LDDDDDGDDTYKEER.- -.LDDDDDGDDTYKEER.- -.LDDDDDGDDTYKEER.- -.PGQAPDGLVDGNKK.- -.M*SAIM*PDQIAK.- -.SFDDLKNIDTK.- -.ISFTTYKPGEVK.- -.M*ISPTGEIGEPGDGDLVSDAFK.- -.M*ISPTGEIGEPGDGDLVSDAFK.-	6.3	5.79	36,645.6	23,502.6	39.91	9	88.25
	YP_001942	Succinate dehydrogenase iron-sulfur subunit (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.NASAM*LFVSAK.- -.VDADVSMDAATCIGCGACVASCK.- -.ITHLGLLPQGK.- -.DISPNM*SFLEM*LDVVNEELITK.- -.DISPNM*SFLEM*LDVVNEELITK.- -.DISPNM*SFLEM*LDVVNEELITK.-	6.3	6.22	35,100.0	26,650.6	25.57	6	60.28
	NP_711213	Enoyl-CoA hydratase (*L. interrogans* serovar Lai str. 56601) -.VAIVADM*GSINR.- -.GINAVYDSPKPSIAAVQK.- -.HCIGGGLDLISACDIR.- -.FFDLILK.- -.FFDLILK.- -.FFDLILK.-	6.3	6.14	35,100.0	30,573.8	19.27	6	60.24

Table 2 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 19 of homogenate of *L. borgpertersenii,* serogroup Tarassovi, serovar Tarassovi separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
7 (con.)	AAS21765	LipL32 (*L. borgpetersenii*) -.LNDDDDGDDTYKEER.- -.SSFVLSESTVPGTNETVK.- -.SSFVLSESTVPGTNETVK.- -.TFLPYGSVINYYGYVKPGQAPDGLVDGNK.- -.TFLPYGSVINYYGYVK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.-	6.3	7.88	35,100.0	28,943.0	34.96	6	54.27
	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) -.PGQAPDGLVDGNK.- -.ISFTTYKPGEVK.- -.AYYLYVWIPAVIAEM*GVR.- -.AYYLYVWIPAVIAEM*GVR.-	6.3	5.74	35,100.0	27,494.1	17.13	4	40.16
	AAV88032	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Javanica) -.TFLPYGSVINYYGYVKPGQAPDGLVDGNK.- -.TFLPYGSVINYYGYVK.- -.TFLPYGSVINYYGYVK.-	6.3	5.37	35,100.0	25,121.4	12.78	3	30.26
	NP_711200	NifU-like protein (*L. interrogans* serovar Lai str. 56601) -.LAGANVEGADFTDAIYDIGTR.- -.LAGANVEGADFTDAIYDIGTR.-	6.3	5.65	35,100.0	29,044.5	7.98	2	20.36
8	AAS21839	OmpL1 (*L. borgpetersenii)* K.PAGEGNYVGVGTR.R K.PAGEGNYVGVGTR.R K.DGLDAATYYGPVR.S	8.3	7.23	35,500.0	29,777.6	9.12	3	20.21
	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) R.ISFTTYKPGEVK.G K.SFDDLKNIDTK.K R.ISFTTYKPGEVK.G	8.3	5.66	35,500.0	27,494.1	9.16	3	20.18
	A40660	Outer membrane protein OmpL1 (*L. alstoni*) K.GAM*VGGNLM*VGYESDFGK.Y	8.3	8.88	35,500	33,530.0	5.63	1	10.30
9	NP_710593	Electron transfer flavoprotein beta subunit (*L. interrogans* serovar Lai str. 56601) -.TAYAM*GADR.- -.ATKEVEGGTQTVETSTPVALTAQK.- -.ATKEVEGGTQTVETSTPVALTAQK.- -.EVEGGTQTVETSTPVALTAQK.- -.EVEGGTQTVETSTPVALTAQK.- -.HGGEVIAVSLGPDR.- -.HGGEVIAVSLGPDR.- -.IEIVGLEPPPPR.- -.WIISPYDEFAIEEGIR.- -.WIISPYDEFAIEEGIR.- -.WIISPYDEFAIEEGIR.-	5.7	5.58	34,841.8	27,084.9	35.97	11	110.27
	AAQ98019	Lip L32 outer membrane protein (*L. interrogans* serovar Icterohaemorrhagiae) -.LDDDDDGDDTYKEER.- -.LDDDDDGDDTYKEER.- -.M*SAIM*PDQIAK.- -.ISFTTYKPGEVK.- -.M*ISPTGEIGEPGDGDLVSDAFK.-	5.7	5.79	34,841.8	23,502.6	21.13	5	50.26
	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) -.QAIAAEESLKK.- -.M*SAIM*PDQIAK.- -.M*ISPTGEIGEPGDGDLVSDAFK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.- -.AYYLYVWIPAVIAEM*GVR.-	5.7	5.74	34,841.8	27494.1	37.05	5	50.19

Table 2 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 19 of homogenate of *L. borgpertersenii,* serogroup Tarassovi, serovar Tarassovi separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
	AAV88032	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Javanica) -.M*ISPTGEIGEPGDGDLLSDAFK.- -.TFLPYGSVINYYGYVKPGQAPDGLVDGNK.- -.TFLPYGSVINYYGYVK.-	5.7	5.37	34,841.8	25,121.4	22.47	3	30.19
10	AAQ98019	Lip L32 outer membrane protein (*L. interrogans* serovar Icterohaemorrhagiae) -.LDDDDDGDDTYKEER.- -.LDDDDDGDDTYKEER.- -.M*SAIM*PDQIAK.- -.ISFTTYKPGEVK.- -.M*ISPTGEIGEPGDGDLVSDAFK.-	6.0	5.79	34,100.0	23,502.6	28.17	5	50.26
	AAS21765	LipL32 (*L. borgpetersenii*) -.LNDDDDGDDTYKEER.- -.TFLPYGSVINYYGYVKPGQAPDGLVDGNK.- -.TFLPYGSVINYYGYVKPGQAPDGLVDGNK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.-	6.0	7.88	34,100.0	28943.0	28.2	4	30.24
11	YP_001491	Flagellin protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.AGDDALGFAM*SEK.- -.LVQLEVDQLIEEVDR.- -.NVM*DGVSFIQVTEGTLEQVNNILQR.-	6.3	6.86	34,841.8	31,551.6	18.60	3	30.26
12	YP_000684	3-oxoacyl-(acyl-carrier-protein) reductase oxidoreductase (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.LDFLVNNAGVLK.- -.LDFLVNNAGVLK.-	6.0	8.33	32,834.5	27,699.7	4.63	2	20.19
13	NP_713477	Elongation factor Ts (*L. interrogans* serovar Lai str. 56601) -.ALEENNADIEK.- -.FGENITIAR.- -.FQVGGL.- -.YVSEVCLVNQAFFK.-	5.5	5.72	31,396.1	22,172.5	20.10	4	40.20
14	YP_002791	Elongation factor Tu (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.AVAYDQIDNAPEEK.- -.LM*EALDTFVPNPK.- -.LM*EALDTFVPNPK.- -.TTLTAAITTTLAK.-	5.7	5.74	32,098.3	43,574.8	9.98	4	40.31
15	YP_001517	tRNA (guanine-N1)-methyltransferase (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.IQSYFSEGLQQK.- -.VDDTIYGGGPGM*LLR.- -.VDDTIYGGGPGM*LLR.-	6.0	5.8	31,743.1	25,594.9	11.69	3	30.18
16	NP_712836	60 kDa chaperonin (*L. interrogans* serovar Lai str. 56601) -.SIETTLDVVEGM*QFDR.- -.TNDVAGDGTTTATILAQSIINEGLK.- -.TNDVAGDGTTTATILAQSIINEGLK.-	5.7	5.29	29,471.8	57,970.4	7.51	3	30.24
	NP_713477	Elongation factor Ts (*L. interrogans* serovar Lai str. 56601) -.ALEENNADIEK.- -.ALEENNADIEK.- -.YVSEVCLVNQAFFK.-	5.7	5.72	29,471.8	22,172.5	12.56	3	30.21
	AAZ73230	Outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.- -.AYYLYVWIPAVIAEM*GVR.-	5.7	5.29	29,471.8	27,494.1	19.52	2	20.19

Table 2 Orthologous proteins of the database matched with the peptide sequences generated from tryptic digestion of proteins in spots no. 1 to 19 of homogenate of *L. borgpertersenii,* serogroup Tarassovi, serovar Tarassovi separated by 2DE at non-linear pH 3-10 (continued)

Spot no.	Accession number	Orthologous proteins(s)/sequences	pI		Molecular mass		Sequence coverage	Matched peptides	Score
			Gel	Theoretical	Gel	Theoretical			
17	YP_001188	peroxiredoxin (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.NIDEAIR.- -.NIDEAIR.- -.M*PQVTSLAPDFK.- -.M*PQVTSLAPDFK.- -.QATINDLPVGR.- -.QATINDLPVGR.- -.YPLIADLTK.-	5.7	5.81	27,262.1	21,517.7	20.21	6	68.19
	AAZ73230	outer membrane lipoprotein LipL32 (*L. interrogans* serovar Sejroe) -.M*SAIM*PDQIAK.- -.M*SAIM*PDQIAK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.- -.GSFVASVGLLFPPGIPGVSPLIHSNPEELQK.- -.AYYLYVWIPAVIAEM*GVR.-	5.7	5.29	27,262.0	27,494.1	23.90	5	50.19
18	YP_002381	hypothetical protein LIC12452 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.LSDLPAPVEVK.- -.LSDLPAPVEVK.- -.SSLDTLAIIAYK.-	6.0	5.11	27,262	20,849.0	12.30	3	30.16
	YP_000728	transcription antitermination protein (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.IPTM*DVAEM*K.- -.FLIQSLPSVSTFVGSK.-	6.0	6.64	27,262	20,455.6	14.36	2	20.17
19	YP_000744	hypothetical protein LIC10764 (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130) -.LESVIDVLVSK.-	6.3	8.87	25,375.9	18,365.1	6.75	1	10.17

group Icterohaemorrhagiae, serovar Icterohaemorrhagiae which is the homologous system was not done in this study because of the inadequate supply of the bacterial cells at the time of study.

Because the complete genome sequences of at least three *Leptospira* serovars, *i.e.* Copenhageni, Lai and Borgpetersenni were established and the information are available in the database,[19-21] thus, identification of the most immunogenic proteins of the two studied *Leptospira* spp. were possible. Nevertheless, there are still few immunogenic proteins, *i.e.* hypothetical proteins that are needed to be elucidated.

For leptospirosis, antibodies are believed to be the immune correlate of protection against and recovery from the infection/disease.[22-24] However, the *Leptospira* spp. grows slowly in the *in vitro* culture with the doubling time of 12-14 hours,[25] rendering inadequate supply of the vaccine raw materials, *i.e.* the whole cells and the native outer membrane proteins. Moreover, the vaccines confer immunity limited only to the homologous or closely related *Leptospira* infection.[26,27] As such, identification of the immunogenic proteins common among the pathogenic *Leptospira* spp. is necessary as they are not only the potential candidates of the vaccine that are likely to protect across serogroups and serovars of *Leptospira* spp. (broad protective spectrum vaccine) but also their coding DNA as well as their recombinant protein counterparts could be more readily prepared than the conventional whole cells and native membrane components.

Among gram negative bacteria, several transmembrane/membrane associated proteins and their functions are known[30] These are for examples: the light absorption-driven transporters (rhodopsin-like protein, light harvesting complex), the oxidoreduction-driven transporters (*e.g.* succinate dehydrogenase, transmembrane cytochrome B-like

Table 3 Functions of the immunogenic proteins of the two pathogenic *Leptospira* spp.

Function of protein	Spot no. (Copenhageni)	Spot no. (Tarassovi)
1. Proteins involved in transcription and translation		
DNA polymerase III, beta subunit	4	-
Transcription antitermination protein	-	18
Elongation factor Tu	4	14
Elongation factor Ts	-	13, 16
tRNA (guanine-N1)-methyltransferase	-	15
2. Proteins functioning as enzymes for metabolisms and nutrient acquisition		
Acetyl-CoA acetyltransferase	3	not done but seen in Fig. 2B
Enoyl-CoA hydratase		7
Dihydrolipoamide succinyltransferase	3	not done but seen in Fig. 2B
Putative glutamine synthetase protein	3	not done but seen in Fig. 2B
Glyceraldehyde-3-phosphate dehydrogenase	5	not done but seen in Fig. 2B
NifU-like protein	-	7
3-oxoacyl-(acyl-carrier-protein) reductase oxidoreductase	-	12
Sphingomyelinase C precursor	2	not done but seen in Fig. 2B
Ssigma factor WhiG	9	
Spermidine synthase	-	5
Succinyl-CoA synthetase beta subunit	4	-
Succinate dehydrogenase iron-sulfur subunit	-	7
3. Proteins/enzymes necessary for energy and electron transfer		
Electron transfer flavoprotein beta-subunit	10, 11	20, 22
Electron transport flavoprotein beta subunit	10, 11	-
Proton-translocating transhydrogenase, subunit alpha part 1	4	-
4. Degradation of misfolded proteins		
ATP-dependent Clp protease, proteolytic subunit	13	-
5. Chaperone		
60 kDa chaperonin	-	3, 16
6. Signal transduction system		
Response regulator	10	-
7. Protein used for host immune evasion		
Peroxiredoxin	-	17
8. Cell structures		
MreB (Cytoskeletal)	5	not done but seen in Fig. 2B
Flagellin	7, 8, 9, 11	2, 11
Periplasmic flagellin	7	-
9. Lipoproteins/Outer membrane proteins		
LipL32	-	3, 4, 5, 6, 7, 8, 9, 10, 16, 17
LipL41	12	1
LipL45	9	-
OmpL1	-	8
10. Hypothetical proteins		
LIC10314	1	-
LIC10483	5, 7	4
LIC10643	12	-
LIC10764		19
LIC11637	11	-
LIC12263	6	not done but seen in Fig. 2B
LIC12452	-	18
LIC13123	10	-
LIC13166	5, 6	not done but seen in Fig. 2B
LIC13477	2	-

proteins, coenzymes–cytochrome C reductase, formate dehydrogenase, cytochrome C oxidases), the electrochemical potential-driven transporters (proton and sodium translocating ATPases), the P-P-bond hydrolysis-driven transporters (calcium ATPase, general secretory pathway translocon, ABC transporter, drug transporter), the porters (uniporters, symporters, antiporters), siderophores, and other α-helical and β-barrels transmembrane proteins.[30] For the pathogenic *Leptospira* spp., the proteins known to be constitutively expressed on the bacterial surface are, for examples, LipL41, LipL32 (Hap1) and trimeric OmpL1 porin proteins. These proteins prepared from the *Leptospira* spp. grown *in vitro* have been tested as the vaccine candidates.[28,29] While the results are encouraging, the inadequate supply of the components limits the vaccine accessibility by the population at the leptospirosis risk. Other integral transmembrane-, membrane associated-and secreted/ recreted- proteins and enzymes also have potential as vaccine candidates provided that they are immunogenic in mammalian hosts.

Our results on the proteomes and immunomes of the pathogenic *Leptospira* spp. should be, more or less, useful information for designing a vaccine, either aiming at inducing humoral and/or cell-mediated immunity, and development of both antibody based- and antigen-based diagnostic assays.

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