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Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis

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Abstract

Leptospirosis is an important global human and veterinary health problem. Humans can be infected by exposure to chronically infected animals and their environment. An important focus of the current leptospiral research is the identification of outer membrane proteins (OMPs). Due to their location, leptospiral OMPs are likely to be relevant in host–pathogen interactions, hence their potential ability to stimulate heterologous immunity. The existing whole-genome sequence of *Leptospira interrogans* serovar Copenhageni offers a unique opportunity to search for cell surface proteins. Predicted genes encoding potential surface proteins were amplified from genomic DNA by PCR methodology and cloned into an *Escherichia coli* expression system. The partially purified recombinant proteins were probed by Western blotting with sera from human patients diagnosed with leptospirosis. Sixteen proteins, out of a hundred tested, were recognized by antibodies present in human sera. Four of these proteins were conserved among eight serovars of *L. interrogans* and absent in the non-pathogenic *Leptospira biflexa*. These proteins might be useful for the diagnosis of the disease as well as potential vaccine candidates.

Keywords: *Leptospira interrogans*; Surface protein; Cloning and expression; Vaccine; Leptospirosis

1. Introduction

Leptospirosis, an emerging infectious disease, is a worldwide zoonosis of human and veterinary concern. Caused by spirochaetes of the genus *Leptospira*, the disease presents greater incidence in tropical and subtropical regions [1,2]. The transmission of leptospirosis has been associated with exposure of individuals in close proximity to wild or farm animals [3]. Recently the

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disease became prevalent in cities with sanitation problems and large population of urban rodent reservoirs, which contaminate the environment through their urine [4]. The incidence of leptospirosis remains underestimated in part due to the broad spectrum of signs and symptoms that patients may present. Children primarily show fever, vomiting, headache, diarrhea, abdominal and generalized muscle pain, whereas adults have fever, headache, anorexia, muscle pain and constipation [4,5]. Five percent to 15% of the cases evolve more severely presenting hemorrhages with renal and hepatic failure, a condition known as Weil's syndrome [4], with a mortality rate of 5–40%. Leptospirosis has also a great economic impact in the agricultural industry since the disease affects the livestock inducing abortions, stillbirths, infertility, reduced milk production and death [3,4].

Currently available veterinarian vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. These types of vaccine confer protective responses through induction of antibodies against leptospiral lipopolysaccharide [6]. However, these vaccines fail to induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation. There is a large number of pathogenic serovars (>200) which imposes a major limitation to the production of a multi-serovar component vaccine and to the development of immunization protocols based on whole cell or membrane preparations. In humans, a prototype vaccine has been tested in China but children under 14 years were not protected [7]. A vaccine licensed for human is still long awaited [4].

The advent of whole-genome sequencing has made an impressive impact in the microbial field landscape. The complete genomic sequence of *Neisseria meningitidis* serogroup B offered a new strategy for the identification of vaccine candidates [8]. This landmark approach, now called reverse vaccinology, has been applied in the last few years revolutionizing the vaccine research area [9,10]. The design of vaccines is based on bioinformatic tools for the prediction of potential antigens *in silico*, hence narrowing down the universe to be tested. In addition, this approach has the advantage of revealing proteins independently of their abundance and without the need of growing the microorganism in vitro [11].

In the present study, we describe 16 new leptospiral membrane-associated proteins selected from the genome of *L. interrogans* serovar Copenhageni [12,13]. The rationale for the choice of these predicted coding sequences is that surface-associated molecules are potential targets for inducing immune responses in animals and may serve as vaccines against disease and/or for use in diagnostic tests.

2. Materials and methods

2.1. Bacteria

L. interrogans serovar Copenhageni sequenced strain (Fiocruz L1-130) was provided by Dr. A.I. Ko (CPqGM/FIOCRUZ/MS). The strain was isolated as described [12]. *L. interrogans* serovars Canicola, Icterohaemorrhagiae, Copenhageni, Bratislava, Hardjo, Autumnalis, Pomona, Pyrogenes, Grippotyphosa and *Leptospira biflexa* serovar Patoc were maintained in one of our laboratories (S.A.V., Faculdade de Medicina Veterinária, Universidade de São Paulo, SP, Brazil).

2.2. In silico *identification of surface proteins*

Protein coding genes from the *L. interrogans* genome sequence were identified using GeneMark and Glimmer [14]. The PSORT program [15] (http://psort.nibb.ac.jp/) was then used to predict the localization of the coded proteins within the bacterium. Public and custom sequence-specific search algorithms were used for identification of sequence motifs including signal peptides, lipoprotein cleavages sites and transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM) [16] and (http://www.cbs.dtu.dk/services/SignalP) [17]. In addition, putative proteins, homologous to surface proteins previously characterized as virulence factors in other organisms, were searched for by blast analysis (http://www.ncbi.nlm.nih.gov/BLAST/) [18]. In this work, the predicted coding sequences are referred to according to their genome nomenclature, LIC [12].

2.3. Cloning, expression and purification

Cloning techniques were performed according to Sambrook et al. [19]. For the expression of the selected predicted coding sequences the Gateway® (Invitrogen) cloning and expression system was used. Each selected DNA sequence was amplified by PCR from *Leptospira* genomic DNA using *Pfx* DNA polymerase (Invitrogen) and primers specially designed following the manufacturer recommendations. In this system, the PCR products are first cloned into pENTR TOPO vector (Invitrogen). The correct orientation is obtained by adding four bases to the forward primer (CACC) which anneals to a complementary overhang in the cloning vector (GTGG). In Table 1, there is a list of primers for PCR amplification of 17 coding sequences which became of special interest in this report. When necessary, DNA bands were extracted from agarose gel using Concert purification system (Gibco BRL) following the manufacture's protocol. The sequence of the cloned leptospira DNA insert was confirmed by sequencing and transferred by recombination reaction by LR Clonase (Invitrogen) to the *Escherichia coli* expression vector

Table 1
Sequence of the primers employed for DNA amplification and molecular mass of native and expressed recombinant proteins

Gene id genome nomenclature*	Primers for PCR amplification	Molecular mass (kDa)	
		Native	Recombinant
LIC10054	F: CACCACGTCTTGTGCGTCGGTAGAG R: CCAAGTATTCTATTTATACGTCCGAG	35.6	35.1
LIC10091	F: CCATGGGACTCGAGACGCCTCCTCCTAAAGATCC R: CTCCATGGTCATTTCAAAACTTCTACGGGGC	40.6	39.0
LIC10508	F: ACCATGGGATCCGCTCTTTTGGTTGATCCAGAG R: GAATTCCTAACAACCAGGACCTTCACAT	23.0	18.5
LIC11947	F: CACCCCTTCGAGGTTGGAAATCG R: AATCGATGGATCACGTTACG	20.1	22.8
LIC10561	F: CACCAAGAAGGATTCCAACGATGATG R: TCTCCTGCTTGACAGCCGAC	33.1	32.0
LIC10765	F: CACCGAAAGTCCCGTAAGGTTCAAA R: TGCAGGAGTTCCCACATTTTA	16.6	15.4
LIC11271	F: CACCAATCGACTTTTCACTGAGTTTCTT R: CGAAAGTATCAAGAAGAACCGTA	30.9	29.2
LIC11574	F: CACCATCATTCCTTCGGGAAGTGAC R: CCATTCTCTGTTGTTTGATCCC	21.2	20.6
LIC12518	F: CACCCCGTGTTCTTTTGGTTTAGAT R: TTCCAACAAATCGAATCATCT	15.8	15.9
LIC13131	F: CACCACGTCTCAAAGTTACGCTTCAG R: TTCTCACCATCCAGCTCGG	23.0	21.9
LIC10380	F: CACCATGGGCGCTTTTAATCGG R: CGGAACTAGGGAACTTTTCAAC	20.9	21.9
LIC12099	F: CACCACCAATGTGTTTGGTATAGCG R: CAGCGTTTTGTGATAAAATTAAC	52.7	53.8
LIC12228	F: CACCAAACCTGGATATGGAATGGC R: TACAGAGGTAGAAGCGTTAGAAG	17.3	18.4
LIC13306	F: CACCTTTGCACAATCCAAAGAGAAATG R: TCATTTCCGAACCGGATGAC	20.1	21.2
LIC10191	F: CACCGAGCCTTCAACGCAAGAGCAA R: AACGTAAGACGTTGAGTTGCCACA	18.7	20.6
LIC13008	F: CACCATGCGTGCTGTCAGTAGAGAAAC R: GTCGACATTGGCAGAATTTACG	15.3	15.2
LIC11352	F: CACCGGTGCTTTCGGTGGTCTG R: ATTACTTAGTCGCGTCAGAAGC	29.6	30.2

* LIC: *Leptospira interrogans* Copenhageni.

pDEST17. The vector containing the correct DNA sequence was cloned into *E. coli* BL21(DE3) (Novagen) for protein expression as previously described [20]. The production of recombinant proteins was achieved by the addition of isopropyl β-D-thiogalactoside (IPTG) to the medium. The pDEST17 vector allows the expression of recombinant proteins with 6 × His-tag at the N-terminus. Most of the assays were performed in high throughput scale with 96 samples each round. The first screening to check the protein expression was done by SDS-PAGE with total protein extracts from each clone. Proteins from expressing clones were purified from the pellets of the cell extracts, by solubilization in 8 M urea, followed by metal affinity chromatography using QIAfilter 96 plates Ni–nitrilotriacetic acid resin superflow (Qiagen) as described by the manufacturer. In brief, contaminants were washed away with 10 vol. of binding buffer, 20 mM Tris, 500 mM NaCl, containing 5 or 20 mM imidazole. Recombinant protein samples were eluted with 5 vol. of the same buffer but with 1 M imidazole. Eluted fractions were analyzed by SDS-PAGE, through 12–15% (wt/vol) acrylamide concentration according to the expected molecular mass of the proteins.

2.4. Mice antisera against recombinant proteins

Groups of 5, 6–8 weeks female BALB/c mice, (Institute Butantan, São Paulo, SP, Brazil) were immunized subcutaneously with approximately 15 μg of each recombinant protein with complete Freund's adjuvant. After 21 days, mice were boosted with the same amount of protein in incomplete Freund's adjuvant. Twenty-eight days following the booster the animals were bled and immune titers were determined by antibody capture endpoint enzyme-linked immunosorbent assay (ELISA). The control group was inoculated with PBS and adjuvant.

2.5. Recognition of recombinant proteins by sera from leptospirosis patients

Cell pellets of induced bacteria as well as purified proteins were separated by 12–15% SDS-PAGE and electro transferred to nitrocellulose membrane for Western blotting assay. Membranes were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% nonfat dry milk; after which they were incubated with individual human serum (microscopic agglutination test – MAT – titre of 25,000, Instituto Adolfo Lutz, Sao Paulo, SP, Brazil) from convalescent patients diagnosed with leptospirosis at 1:100 dilution. Goat anti-human IgG peroxidase-conjugate was used as secondary antibody at 1:1000 dilution in PBS-T. Proteins reacting with the antisera were detected by reaction with DAB and H_2O_2 or with ECL Kit (Amersham).

2.6. Recognition of proteins from leptospiral extracts by sera from mice immunized with recombinant proteins

Bacterial cultures of several leptospiral serovars were centrifuged and the cell pellets were resuspended and washed three times by centrifugation with PBS containing 5 mM $MgCl_2$. The cell pellets were then resuspended in PBS containing 10% SDS and a sample from each serovar sample was applied on a 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with serum obtained from mice immunized with each of the recombinant proteins.

2.7. Nucleotide sequence Accession Numbers

The sequences of the two chromosomes of *L interrogans* serovar Copenhageni strain Fiocruz L1-130 have been deposited in the GenBank database under Accession Nos. AE016823 and AE016824. The accession numbers for public data base for each protein sequence analyzed in this work are listed in Table 2. The proteins can also be accessed by the genome nomenclature for the gene locus, LIC number (*Leptospira interrogans* Copenhageni).

3. Results

3.1. Identification of vaccine candidates from the leptospiral genome: in silico analysis

Our rationale for the selection of the predicted coding sequences is that surface associated molecules are potential targets for inducing immune responses in the host [8–11]. These sequences could be identified in the genome database by one or more sequence motifs commonly found in known surface proteins from other bacteria. A primary screening was performed using the Psort program [http://psort.nibb.ac.jp/] to identify putative proteins with predicted cellular localization from the inner to the outer bacterial membrane. Genes encoding proteins with known cytoplasmic functions were excluded. In addition, we searched for exportation signal peptides, transmembrane domains, lipoprotein signatures and homologies to known surface proteins [12]. The *in silico* approach resulted in a large number of genes covering ~20% of the total number of predicted proteins in the genome. From these sequences we focused the selection mainly on hypothetical, unknown proteins, having either signal peptide sequences or lipobox motifs [12].

Table 2
Name, feature and accession number of the proteins reactive to sera of leptospirosis patients

Locus id	Protein name*	Feature	Protein Accession No.
LIC10054	MPL36	Lipoprotein, probable**	AAS68691
LIC10091	LipL40	Lipoprotein, probable**	AAS68725
LIC10508	LipL23	Lipoprotein, probable**	AAS69129
LIC11947	LipL22	Lipoprotein, probable**	AAS70529
LIC10561	OMPL31	Leptospira conserved hypothetical protein**	AAS69182
LIC10765	MPL17	Leptospira conserved hypothetical protein	AAS69382
LIC11271	OMPL30	Leptospira conserved hypothetical protein	AAS69877
LIC11574	OMPL22	Leptospira conserved hypothetical protein	AAS70170
LIC12518	OMPL16	Leptospira conserved hypothetical protein	AAS71083
LIC13131	MPL21	Leptospira conserved hypothetical protein	AAS71676
LIC10380	OMPL21	Conserved hypothetical protein	AAS69003
LIC12099	LipL53	Conserved hypothetical protein**	AAS70670
LIC12228	OMPL17	Conserved hypothetical protein	AAS70800
LIC13306	OMPL20	Conserved hypothetical protein	AAS71848
LIC10191	OMPAL21	Membrane protein, peptidoglycan associated (OmpA-like)**	AAS68819
LIC13008	OMPL15	Hypothetical protein	AAS71558
LIC11352	LipL32	Lipoprotein LipL32	AAS69953

* MP, Lip and OMP stand for membrane protein, lipoprotein and outer membrane protein, respectively; OMPA stands for OMPA-like domain; L, refers to *Leptospira*, while the numbers are related to the molecular mass of the protein.
** Probable lipoproteins according to criteria described in Nascimento et al. [12] and Haake [22].

3.2. Cloning and expression of vaccine candidates

Oligonucleotides for PCR amplifications (Table 1) were designed from the genome without the signal peptide sequences, which typically contain 18–28 nucleotides, as described in experimental procedures. From 206 selected coding sequences (see Table 1, supplementary data), more than 97% were amplified. The correct sequences were confirmed by DNA sequencing and 175 genes (84%) were successfully cloned into pENTR. The DNA inserts were transferred by recombination from pENTR to pDEST17 expression vector. This *E. coli* vector expresses the recombinant proteins with six histidine residues at the N-terminus which allows a rapid purification of the protein by metal chelating chromatography. We have expressed and purified 150 recombinant proteins with this system. A representative set of SDS-PAGE containing cell extracts from induced *E. coli* BL21(DE3) cultures expressing the recombinant proteins is shown in Fig. 1. Based on the results obtained with the immune assays (see below), 16 proteins were found to be of special interest and the data related to them are highlighted along this report. Some of these proteins are indicated in Fig. 1.

The majority of the recombinant proteins were found to be insoluble in the pellets of bacterial cell lysates, as evaluated by SDS-PAGE (not shown). The inclusion bodies were isolated, urea-solubilized and purified by metal affinity. The purified proteins were grouped according to their molecular mass and analyzed by SDS-PAGE. A representative set of these gels is shown in Fig. 2, in which numbered lanes refer to proteins reactive to sera of leptospirosis patients (see below).

3.3. Screening recombinant proteins for reactivity with leptospiral antisera

Purified proteins were screened for reactivity by immunoblotting with pooled sera from patients diagnosed with leptospirosis. The recombinant lipoprotein LipL32 was used as a positive control since it was shown to be immunogenic and highly conserved among *Leptospira* pathogenic serovars [20,21]. A total of 16 proteins out of a hundred tested were recognized by sera from leptospirosis's convalescent patients (Fig. 3). Table 2 summarizes features of these sera-reactive recombinant proteins.

3.4. Features of the proteins recognized by antibodies

The recombinant proteins listed in Table 2 were recognized by human sera from patients diagnosed with leptospirosis. They are all predicted to be membrane proteins with a signal peptide of 20–36 amino acids, as indicated by the software described in Bendtsen et al. [17]. Seven of these proteins (indicated by ** in Table 2) are probably new leptospiral lipoproteins according to the criteria previously described [12,22]. The signal peptides regions are characterized by a higher proportion of hydrophobic amino acids, a lipoprotein signal peptidase and a cysteine to be lipidated [22]. No specific function could be attributed to these putative lipoproteins. LIC10054 encodes a rare lipoprotein domain to which homologs were already found in other microorganisms according to GeneBank database [http://www.ncbi.nlm.nih.gov/BLAST/] [18]. Interestingly, the protein named OMPL15 (LIC13008) did not match any sequence in the available protein databases and should be considered for further studies on protein function. The protein encoded by LIC10191 has been

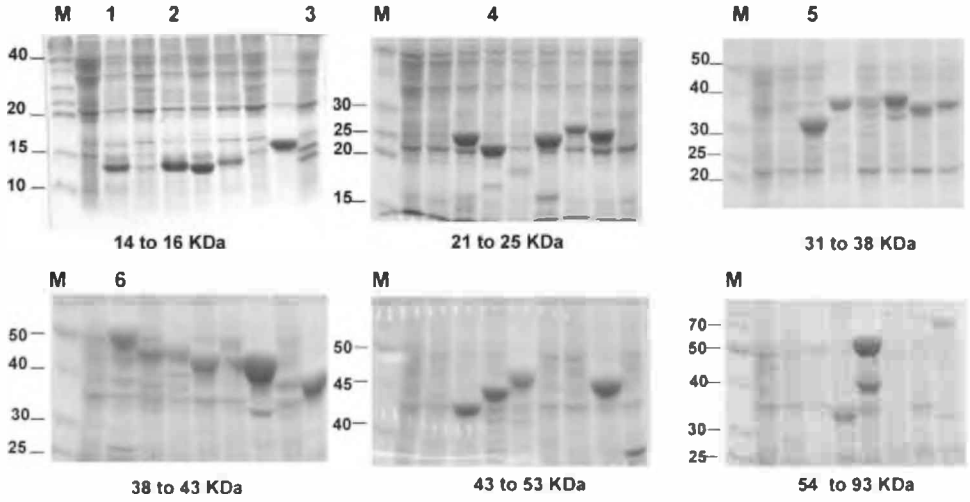

Fig. 1. Protein expression analysis by SDS-PAGE. Representative gels showing the cell extracts of BL21(DE3) *E. coli* transformed with different expression vectors. Proteins are assembled according to their expected molecular mass. Lane numbers refer to sera-reactive proteins: (1) OMPL16, (2) OMPL15, (3) MPL17, (4) LipL22, (5) OMPL31 and (6) LipL53. **M** indicates the protein molecular mass marker.

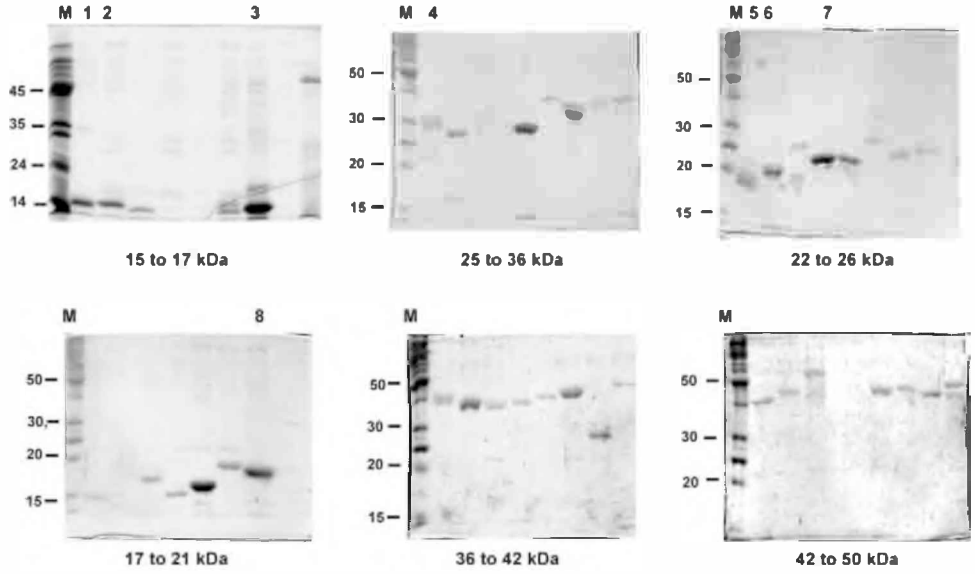

Fig. 2. Purification of recombinant proteins. Representative SDS-PAGE containing samples of purified proteins obtained by high throughput metal affinity chromatography purification system. Proteins are assembled in the gels according to their expected molecular mass. Lane numbers refer to sera-reactive proteins: (1) OMPL15, (2) MPL17, (3) OMPL16, (4) OMPL30, (5) MPL21, (6) OMPL21, (7) LipL22, and (8) OMPL22. **M** indicates the protein molecular mass marker.

recently characterized from *L. interrogans* serovar Manilae, strain UP-MMC, as a novel lipoprotein with an OmpA domain [23]. Three proteins out of the 16 were found to be hypothetical proteins conserved among other microorganisms, while five were hypothetical proteins conserved only among *Leptospira* species (Table 2), according to the currently available databases.

3.5. Conservation of antigenic proteins among leptospiral serovars

Protein expression in the most prevalent pathogenic serovars of *L. interrogans* is an important requirement for leptospiral vaccine candidates. In order to characterize the conservation of the selected proteins we employed protein extracts from several *L. interrogans* serovars: Canicola, Icterohaemorrhagiae, Copenhageni, Bratislava, Hardjo, Autumnalis, Pomona, Pyrogenes, Grippotyphosa and the nonpathogenic strain *L. biflexa* serovar Patoc. Cell extracts were prepared from cultures of the different serovars and Western blot analysis was performed with polyclonal sera from mice immunized with each recombinant protein. The serological cross-reactivity showed a high degree of conservation among four out of 10 proteins tested (Fig. 4). Interestingly, none of these four proteins were present in the nonpathogenic *L. biflexa* strain, suggesting that they may be relevant for pathogenesis. Most of the proteins reac-

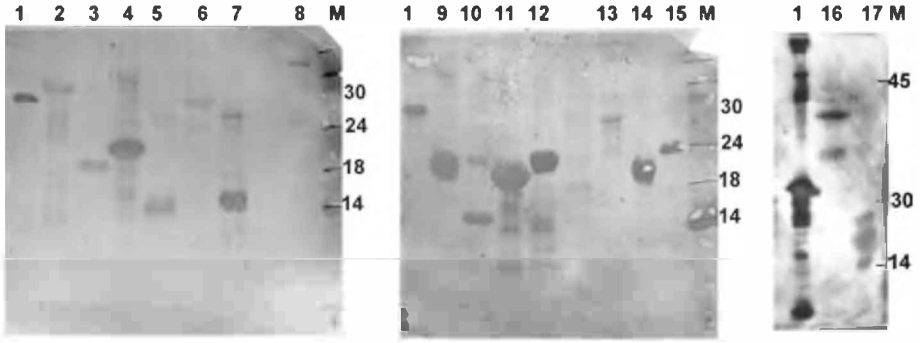

Fig. 3. Recognition of recombinant proteins by leptospirosis human serum. Proteins blotted into nitrocellulose membranes were probed with serum (1:100 dilution) from individual covalescent patient diagnosed with leptospirosis (microscopic agglutination test – MAT – titre of 25,000, Instituto Adolfo Lutz, Sao Paulo, SP, Brazil). Anti-human IgG-peroxidase conjugate was used as the secondary antibody and the bands were developed with DAB/H_2O_2. Lanes: 1. LipL32 (30.2); 2. LipL40 (39.0); 3. LipL23 (18.5); 4. OMPL20 (21.2); 5. OMPL15 (15.2); 6. OMPL31 (32.0); 7. OMPL16 (15.9); 8. LipL53 (53.8); 9. MPL21(21.9); 10. MPL17 (15.4); 11. OMPL22 (20.6); 12. OMPL21 (21.9); 13. OMPL30 (29.2); 14. OMPL17 (18.4); 15. LipL22 (22.8); 16. MPL36 (35.1) and 17. OMPAL21 (20.6). Numbers in parenthesis are the molecular mass of the recombinant proteins. **M** indicates the protein molecular mass marker.

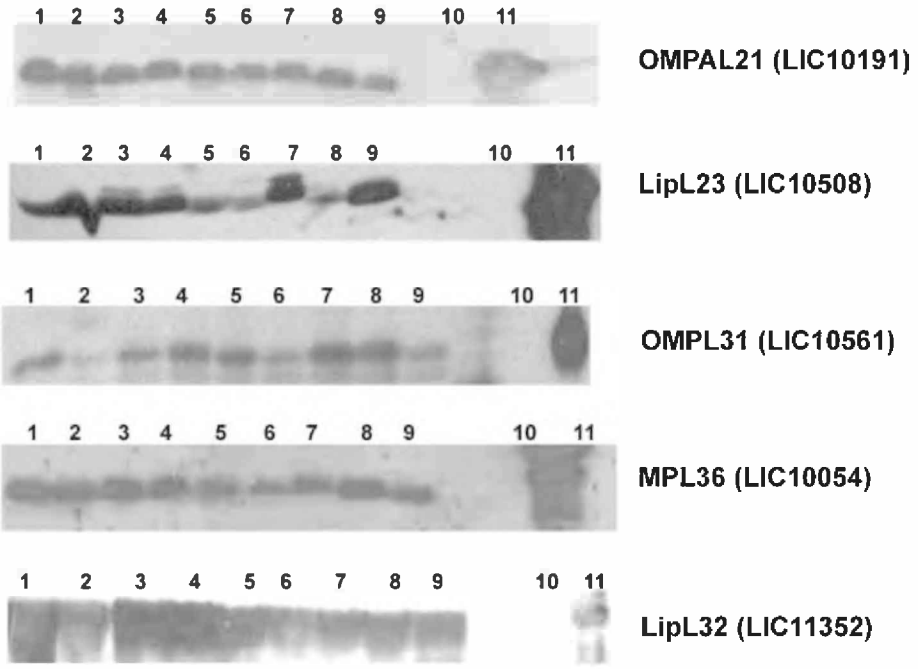

Fig. 4. Conservation of proteins among pathogenic leptospires. Whole cell lysates were prepared from representative strains of *L. interrogans* serovars. Proteins from the lysates were separated by electrophoresis, transferred into nitrocellulose membranes and probed with mice antisera raised against the recombinant proteins, as indicated by protein name and LIC number. Serovars in lanes are: 1. Canicola; 2. Icterohaemorrhagiae; 3. Copenhageni; 4. Bratislava; 5. Hardjo; 6. Autumnalis; 7. Pomona; 8. Pyrogenes; 9. Grippotyphosa; 10. *L. biflexa* sv Patoc; 11. respective purified recombinant protein (positive control).

tive with human leptospirosis serum shared roughly comparable expression levels among the different serovars tested, but LIC10508 and LIC10561 were more variable.

4. Discussion

Considering that *L. interrogans* has an AT rich genome, with a 35% G + C content, [12], which imposes certain difficulties for primer design, it was above expectations to have amplified more than 97% of the 206 coding sequences, as well as to have expressed and purified more than 80% of the cloned products. By screening with immune sera from leptospirosis patients 16 proteins were identified as potential vaccine candidates or to be used in diagnosis. The fact that these proteins react with infected human sera not only shows their immune reactive properties, but also strongly suggests that these proteins are expressed in the course of human infection. Interestingly, one of them (OMPL15) did not match at all any protein sequence in the public databases and deserves further studies to characterize its origin and function.

The central mechanism in pathogenesis of leptospirosis, as in other spirochetal diseases such as Lyme disease and syphilis, is the ability of the pathogens to disseminate widely within the host during the early stage of infection [4]. Although surface-associated proteins that play a role in virulence have not been yet identified, it is assumed that they may mediate interactions that enable entry and dissemination through host tissues. Putative outer membrane proteins would be accessible to the immune response during host infection and therefore, constitute targets for immune protection through mechanisms such as antibody-dependent phagocytosis and killing mediated by complement. In this regard, a major limitation in the field of leptospirosis has been to identify membrane associated proteins through conventional biochemical and molecular methods. For instance, before the complete genome sequence of a leptospira was known, only 10 lipoproteins had been characterized through isolation of membrane fractions [24–29]. Using the genome approach, we have identified 174 novel putative lipoproteins [12]. In addition, the identified leptospiral proteins may also have diagnostic applications. At present, rapid and efficient diagnostic tests are not available for use in human and veterinary leptospirosis [1,2,4]. A proportion of these membrane proteins will be antigens expressed during infection and should be recognized by the host immune system. Therefore, they may serve as the basis to develop antigen-capture detection strategies or recombinant protein-based serologic tests.

Our results corroborate previous studies showing the advantages of using whole genome approach [10,11] for

the identification of novel vaccine antigens at a reasonable cost and time compared with traditional strategies.

We believe this work represents an important contribution to the leptospiral field. The proteins described herein may certainly be exploited for the establishment of a much-needed kit for diagnosis of leptospirosis. Furthermore, these proteins may provide an optimal basis for the development of a new and effective vaccine that would help reduce the burden of leptospirosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.femsle.2005.02.004.

References

[1] Levett, P.N. (2001) Leptospirosis. Clin. Microbiol. Rev. 14, 296–326.

[2] Levett, P.N. (2003) Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin. Infect. Dis. 36, 447–452.

[3] Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P.N., Gilman, R.H., Willig, M.R., Gotuzzo, E. and Vinetz, J.M. (2003) Peru-United States Leptospirosis Consortium, Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. 3, 757–771.

[4] Faine, S., Adler, B., Bolin, C. and Perolat, P. (1999) *Leptospira* and Leptospirosis, 2nd edn. MediSci, Melbourne, Australia.

[5] Plank, R. and Dean, D. (2000) Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. Microbes Infect. 2, 1265–1276.

[6] de la Pena-Moctezuma, A., Bulach, D.M., Kalambaheti, T. and Adler, B. (1999) Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. FEMS Microbiol. Lett. 177, 319–326.

[7] Zhuo, J.T., Wang, S.S. and Lan, W.L. (1995) A discussion on setting up target age group for imunization against leptospirosis. Zhonghua Liu Xing Bing Xue Za Zhi 16, 228–230.

[8] Pizza, M., Scarlato, V., Masignani, V., Giuliani, M.M., Arico, B., Comanducci, M., Jennings, G.T., Baldi, L., Bartolini, E., Capecchi, B., Galeotti, C.L., Luzzi, E., Manetti, R., Marchetti, E., Mora, M., Nuti, S., Ratti, G., Santini, L., Savino, S., Scarselli, M., Storni, E., Zuo, P., Broeker, M., Hundt, E., Knapp, B., Blair, E., Mason, T., Tettelin, H., Hood, D.W., Jeffries, A.C., Saunders, N.J., Granoff, D.M., Venter, J.C., Moxon, E.R., Grandi, G. and Rappuoli, R. (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science 287, 1816–1820.

[9] Wizemann, T.M., Heinrichs, J.H., Adamou, J.E., Erwin, A.L., Kunsch, C., Choi, G.H., Barash, S.C., Rosen, C.A., Masure, H.R., Tuomanen, E., Gayle, A., Brewah, Y.A., Walsh, W., Barren, P., Lathigra, R., Hanson, M., Langermann, S., Johnson, S. and Koenig, S. (2001) Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. Infect. Immun. 69, 1593–1598.

[10] Rappuoli, R. (2001) Reverse vaccinology, a genome-based approach to vaccine development. Vaccine 19, 2688–2691.

[11] Adu-Bobie, J., Capecchi, B., Serruto, D., Rappuoli, R. and Pizza, M. (2003) Two years into reverse vaccinology. Vaccine 21, 605–610.

[12] Nascimento, A.L.T.O., Ko, A.I., Martins, E.A., Monteiro-Vitorello, C.B., Ho, P.L., Haake, D.A., Verjovski-Almeida, S., Hartskeerl, R.A., Marques, M.V., Oliveira, M.C., Menck, C.F., Leite, L.C., Carter, H., Coutinho, L.L., Degrave, W.M., Dellagostin, O.A., El-Dorry, H., Ferro, E.S., Ferro, M.I., Furlan, L.R., Gamberini, M., Giglioti, E.A., Goes-Neto, A., Goldman, G.H., Goldman, M.H., Harakava, R., Jeronimo, S.M., Junqueira-de-Azevedo, I.L., Kimura, E.T., Kuramae, E.E., Lemos, E.G., Lemos, M.V., Marino, C.L., Nunes, L.R., de Oliveira, R.C., Pereira, G.G., Reis, M.S., Schriefer, A., Siqueira, W.J., Sommer, P., Tsai, S.M., Simpson, A.J., Ferro, J.A., Camargo, L.E., Kitajima, J.P., Setubal, J.C. and Van Sluys, M.A. (2004) Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J. Bacteriol. 186, 2164–2172.

[13] Nascimento, A.L.T.O., Verjovski-Almeida, S., Van Sluys, M.A., Monteiro-Vitorello, C.B., Camargo, L.E., Digiampietri, L.A., Harstkeerl, R.A., Ho, P.L., Marques, M.V., Oliveira, M.C., Setubal, J.C., Haake, D.A. and Martins, E.A.L. (2004) Genome features of *Leptospira interrogans* serovar Copenhageni. Braz. J. Med. Biol. Res. 37, 459–477.

[14] Delcher, A.L., Harmon, D., Kasif, S., White, O. and Salzberg, S.L. (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res. 27, 4636–4641.

[15] Nakai, K. and Kanehisa, M. (1991) Expert system for predicting protein localization sites in gram-negative bacteria. Proteins 11, 95–110.

[16] Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a Hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567–580.

[17] Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

[18] Altschul, F.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402.

[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Plainview, NY.

[20] Dey, S., Mohan, C.M., Kumar, T.M., Ramadass, P., Nainar, A.M. and Nachimuthu, K. (2004) Recombinant LipL32 antigen-based single serum dilution ELISA for detection of canine leptospirosis. Vet. Microbiol. 103, 99–106.

[21] Haake, D.A., Suchard, M.A., Kelley, M.M., Dundoo, M., Alt, D.P. and Zuerner, R.L. (2004) Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J. Bacteriol. 186, 2818–2828.

[22] Haake, D.A. (2000) Spirochaetal lipoproteins and pathogenesis. Microbiology 146, 1491–1504.

[23] Koizumi, N. and Watanabe, H. (2003) Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. FEMS Microbiol. Lett. 226, 215–219.

[24] Haake, D.A., Chao, G., Zuerner, R.L., Barnett, J.K., Barnett, D., Mazel, M., Matsunaga, J., Levett, P.N. and Bolin, C.A. (2000)

The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68, 2276–2285.

[25] Haake, D.A., Martinich, C., Summers, T.A., Shang, E.S., Pruetz, J.D., McCoy, A.M., Mazel, M.K. and Bolin, C.A. (1998) Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect. Immun. 66, 1579–1587.

[26] Haake, D.A. and Matsunaga, J. (2002) Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infect. Immun. 70, 4936–4945.

[27] Haake, D.A., Mazel, M.K., McCoy, A.M., Milward, F., Chao, G., Matsunaga, J. and Wagar, E.A. (1999) Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect. Immun. 67, 6572–6582.

[28] Shang, E.S., Exner, M.M., Summers, T.A., Martinich, C., Champion, C.I., Hancock, R.E. and Haake, D.A. (1995) The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infect. Immun. 63, 3174–3181.

[29] Shang, E.S., Summers, T.A. and Haake, D.A. (1996) Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect. Immun. 64, 2322–2330.