

Evolutionary Implication of Outer Membrane Lipoprotein-Encoding Genes *ompL1*, *lipL32* and *lipL41* of Pathogenic *Leptospira* Species

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Leptospirosis is recognized as the most widespread zoonosis with a global distribution. In this study, the antigenic variation in *Leptospira interrogans* and *Leptospira borgpetersenii* isolated from human urine and field rat kidney was preliminarily confirmed by microscopic agglutination test using monoclonal antibodies, and was further subjected to amplification and identification of outer membrane lipoproteins with structural gene variation. Sequence similarity analysis revealed that these protein sequences, namely OmpL1, LipL32 and LipL41, showed no more homologies to outer membrane lipoproteins of non-pathogenic *Leptospira* and other closely related Spirochetes, but showed a strong identity within *L. interrogans*, suggesting intra-specific phylogenetic lineages that might be originated from a common pathogenic leptospiral origin. Moreover, the *ompL1* gene showed more antigenic variation than *lipL32* and *lipL41* due to less conservation in secondary structural evolution within closely related species. Phylogenetically, *ompL1* and *lipL41* of these strains gave a considerable proximity to *L. weilii* and *L. santarosai*. The *ompL1* gene of *L. interrogans* clustered distinctly from other pathogenic and non-pathogenic leptospiral species. The diversity of *ompL* genes has been analyzed and it envisaged that sequence-specific variations at antigenic determinant sites would result in slow evolutionary changes along with new serovar origination within closely related species. Thus, a crucial work on effective recombinant vaccine development and engineered antibodies will hopefully meet to solve the therapeutic challenges.

Key words: *Leptospira*, *ompL1*, *lipL32*, *lipL41*, phylogeny, antigenic variation

Introduction

Leptospirosis is now recognized as the most widespread zoonosis with a global distribution, which is caused by *Leptospira*, a genus of spirochetal bacteria, either through direct contact of infected animals or through urine (1). An international survey of human leptospirosis conducted by World Health Organization reported that approximately 100,000 severe cases (requiring hospitalization) occur annually (2). Infection with host-adapted leptospiral serovars can result in lifelong renal carriage and urinary shedding. In humans, contact to infected host animals and contaminated water/soil results in potentially lethal

disease. Although many organs could be affected, lung and kidney have been commonly identified as potential targets to be invaded by the leptospire. In obligate intracellular bacteria, the outer membrane proteins (OMPs) play a crucial role in the process of adaptation by facilitating interactions between bacterial cells and its host (3). Though 258 serovars in the genus *Leptospira* have been identified, they are antigenically very distinct due to the lipopolysaccharide (LPS) and spatial arrangement of outer membrane lipoproteins (OmpL), particularly OmpL1, LipL32 and LipL41. In addition, such extensive serovar di-

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versity with distinct antigenic determinant has been attributed to the structural composition of LPS (4–6) and the genetics of LPS biosynthesis (7, 8). Although leptospiral LPS can elicit protective immunity, this immunity is generally serovar specific. Consequently, the focus of research on protective antigens has been shifted towards the identification of conserved OMPs, which may be able to stimulate heterologous immunity.

OMP is considered to play a role in the structural integrity of the organism and is surface-exposed and glycosylated (9). The most abundant class comprises the lipoproteins designated according to their apparent molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, including the major OMP (MOMP) and immunodominant protein antigen LipL32 (10, 11), the *in vivo* down-regulated proteins LipL32 (10) and LipL36 (12), the surface-exposed proteins LipL41 (13, 14) and LipL21 (15). Among these, OmpL1, LipL32 and LipL41 are major antigens in the humoral immune response to leptospirosis and immunology-based diagnostics (16–18).

Comparative analysis of *ompL* gene sequences can reveal insights into a novel mechanism of molecular evolution in pathogenic bacteria. The 16S rRNA genes are the most conserved, followed by the *lipL32*, *lipL41* and *ompL1* genes, listed in the order of increasing sequence variability. The differences in sequence variability persist when synonymous mutations are considered. However, relatively little is known about the mechanisms and molecular diversity extent of leptospiral *ompL* genes (11). Accordingly, an evolutionary examination of the *ompL* genes may enlighten the actual role of OMPs in the pro-

cesses of infection, humoral immunity, and speciation of these organisms. The purpose of the present study is to conclude the phylogenetic relationships of *ompL* genes of some leptospiral strains isolated from human urine and rat kidney with Leptospiraceae homologous sequences in order to better realize their molecular diversity and evolution, which ultimately increases the scope of developing a broad-spectrum OMP-specific vaccine antigen for protective immunity.

Results

The amplified *ompL1*, *lipL32* and *lipL41* genes were viewed at 1% agarose gel and obtained in 960 bp, 782 bp and 1,065 bp, respectively (**Figure 1**); these products were then used for sequencing. A preliminary phylogenetic analysis of similar gene and protein sequences collected from an NCBI-BLAST search indicated that a substantial number of gene and protein sequences were identified as OmpL1, LipL32 and LipL41-like proteins, but they were widely distributed in different serovars of *Leptospira interrogans*, and secondly in *Leptospira borgpetersenii*. Gene and protein sequence similarity analysis (on the basis of e-value and identity score) implied that 96%–99% gene and 90%–95% protein sequence identities have been obtained to *ompL* sequences of *L. interrogans* and *L. borgpetersenii*, on which most of the sequences showed identity within *L. interrogans*. Similarity search tool BLASTn was used to identify similar sequences and the retrieved information is comprehensively summarized in **Tables 1–3**. These data clearly evidenced that there is strong sequence conservation in these genes, which belongs to *L. interrogans* specific, but

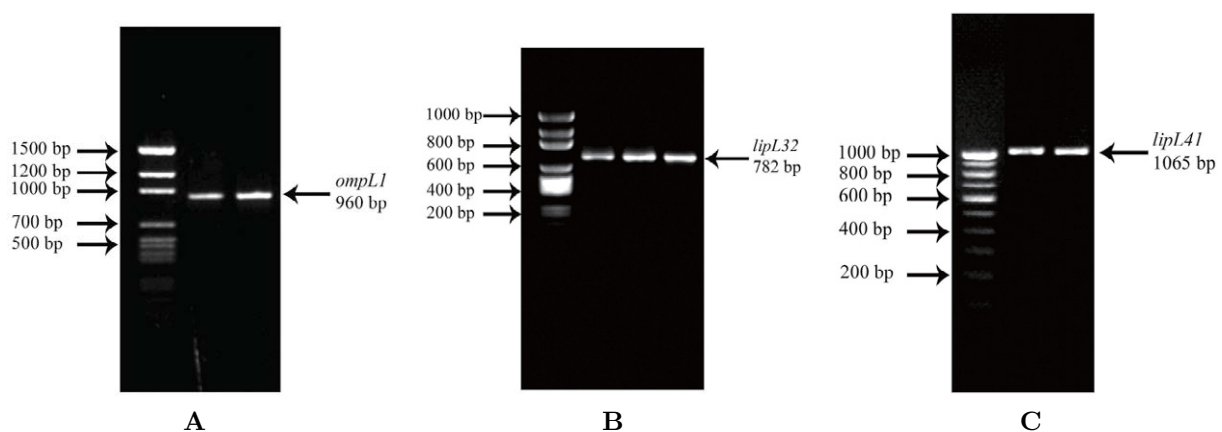


Figure 1 Agarose gel electrophoresis of *ompL* genes. **A.** *ompL1* genes of *L. interrogans* Grippotyphosa CH31 and *L. interrogans* Autumnalis N2. **B.** *lipL32* genes of *L. borgpetersenii* Javanica R1R, R1L and *L. interrogans* Autumnalis N2. **C.** *lipL41* genes of *L. interrogans* Grippotyphosa CH31 and *L. interrogans* Autumnalis N2.

Table 1 BLAST search results of *ompL1* genes of *Leptospira* used for phylogenetic analysis

Organism	Serovar	Strain	Accession No.	Country
<i>L. interrogans</i>	Grippotyphosa	CH31	EU091293	India
<i>L. interrogans</i>	Autumnalis	N2	EU091294	India
<i>L. interrogans</i>	Lai	017	AF255308	China
<i>L. interrogans</i>	Lai	56601	AF250318	China
<i>L. interrogans</i>	Copenhageni	L1-130	AY461983	USA
<i>L. interrogans</i>	Lai	56601	AY461984	USA
<i>L. interrogans</i>	Icterohaemorrhagiae	RGA	AY461985	USA
<i>L. interrogans</i>	Lai	Lai	AY622661	China
<i>L. interrogans</i>	Hebdomadis	P 7	AY622665	China
<i>L. interrogans</i>	Australis	65-9	AY622664	China
<i>L. interrogans</i>	Autumnalis	Lin 4	AY622658	China
<i>L. interrogans</i>	Canicola	Lin	AY622662	China
<i>L. interrogans</i>	Grippotyphosa	Lin 6	AY622659	China
<i>L. interrogans</i>	Wolffi	L 183	AY622660	China
<i>L. interrogans</i>	Pyrogens	Tian	AY622663	China
<i>L. interrogans</i>	Paidjan	L 37	AY622666	China
<i>L. kirschnerii</i>	Grippotyphosa	RM52	AY461991	USA
<i>L. nochugii</i>	Pomona	Luo	AY622667	China
<i>L. weilii</i>	Manhao II	L 105	AY622668	China

Table 2 BLAST search results of *lipL32* genes of *Leptospira* used for phylogenetic analysis

Organism	Serovar	Strain	Accession No.	Country
<i>L. borgpetersenii</i>	Javanica	R1L	EU293441	India
<i>L. borgpetersenii</i>	Javanica	R1R	EU293442	India
<i>L. interrogans</i>	Autumnalis	N2	EU293443	India
<i>L. interrogans</i>	Hardjo	–	AY442332	India
<i>L. interrogans</i>	Wolffi	L 183	AY609332	China
<i>L. interrogans</i>	Paidjan	L 37	AY609329	China
<i>L. interrogans</i>	Canicola	Lin	AY609321	China
<i>L. interrogans</i>	Autumnalis	Lin 4	AY609324	China
<i>L. interrogans</i>	Australis	65-9	AY609325	China
<i>L. interrogans</i>	Grippotyphosa	Lin 6	AY609327	China
<i>L. interrogans</i>	Hebdomadis	P 7	AY609328	China
<i>L. noguchii</i>	Pomona	Luo	AY609326	China
<i>L. borgpetersenii</i>	Mini	Nan 10	AY609333	China
<i>L. interrogans</i>	Pyrogenes	Tian	AY609323	China
<i>L. borgpetersenii</i>	Tarassovi	55-52	AY609330	China
<i>L. borgpetersenii</i>	Ballum	Pishu	AY609322	China
<i>L. borgpetersenii</i>	–	M 10	AY568680	China
<i>L. interrogans</i>	Canicola	Hond Utrecht	AJ580493	India
<i>L. interrogans</i>	Copenhageni	L1-130	AF245281	USA
<i>L. weilii</i>	Manhao II	L 105	AY609331	China

Table 3 BLAST search results of *lipL41* genes of *Leptospira* used for phylogenetic analysis

Organism	Serovar	Strain	Accession No.	Country
<i>L. interrogans</i>	Autumnalis	N2	EU091295	India
<i>L. interrogans</i>	Grippotyphosa	CH31	EU091296	India
<i>L. interrogans</i>	Wolffi	L 183	AY622686	China
<i>L. weilii</i>	Manhao II	L 105	AY622685	China
<i>L. borgpetersenii</i>	Tarassovi	55-52	AY622684	China
<i>L. interrogans</i>	Paidjan	L 37	AY622683	China
<i>L. interrogans</i>	Hebdomadis	P 7	AY622682	China
<i>L. interrogans</i>	Grippotyphosa	Lin 6	AY622681	China
<i>L. noguchii</i>	Pomona	Luo	AY622680	China
<i>L. interrogans</i>	Australis	65-9	AY622679	China
<i>L. interrogans</i>	Autumnalis	Lin 4	AY622678	China
<i>L. interrogans</i>	Pyrogens	Tian	AY622677	China
<i>L. borgpetersenii</i>	Ballum	Pishu	AY622676	China
<i>L. interrogans</i>	Canicola	Lin	AY622675	China
<i>L. borgpetersenii</i>	Javanica	M 10	AY622674	China
<i>L. interrogans</i>	Lai	Lai	AY622673	China
<i>L. interrogans</i>	Canicola	–	AY642287	India
<i>L. interrogans</i>	Hardjo	–	AY642286	India
<i>L. interrogans</i>	Hebdomadis	Akiyami B	AB240675	Japan
<i>L. interrogans</i>	Australis	Akiyami C	AB240676	Japan
<i>L. interrogans</i>	Icterohaemorrhagiae	RGA	AB240677	Japan
<i>L. interrogans</i>	Manilae	UP-MMC	AB240678	Japan
<i>L. interrogans</i>	Autumnalis	Akiyami A	AB240674	Japan

not serovar specific. Only a few sequence substitutions including transition or transversion could result in such close variation among *ompL* genes to bring a new serovar of the same species.

Multiple sequence alignments (MSAs) have been proved to be of high quality, with few insertions or deletions. Results obtained from MSAs of selected leptospiral strains indicated that there were short ranges of sequence variation within the conserved domain of these genes, including deletion or insertion, which brought a major diversification in this family. Moreover, unlike other *ompL* genes of *L. interrogans*, *L. kirschneri* and *L. weilii*, a variation in stretch of nucleotides of these genes has highly influenced on the phylogenetic relationship of our strains. Therefore, it resulted in a separate cluster in the phylogenetic trees as shown in **Figures 2–4**. Even in the regions with gaps, the alignment showed high regions of similarity, and most of the large gaps occurred due to only a few sequences that had long insertions relative to others. We also found greater numbers of indels and a high level of dissimilarity when comparing the alignment of these genes, confirming the appropriateness of these

sequences as our groups. Comparative sequence analysis of OMPs has provided information about the variability of *ompL*-like genes found in *Leptospira*, suggesting the probability of slow evolutionary changes among serotypes of even the same species that can elicit immunogenicity and host infection (**Figure 5**).

We used a neighbor-joining (NJ) analysis and the underlying pairwise distance matrix to remove redundant sequences and to select a set of *ompL* representing the greatest diversity in terms of both sequence and genome diversity for phylogenetic analysis. The *ompL1*, *lipL32* and *lipL41* sequences formed monophyletic groups with posterior probabilities of 1.0, 0.9 and 0.79, respectively, and high NJ bootstrap support of 90%–95%. Both minimum evolution (ME) and NJ analyses produced similar trees, with some discrepancy in branching order of *lipL32* gene. These analyses produced trees that segregated the serovars of *L. interrogans* into four clusters consistent with different serotypes. The phylogenetic analyses also provided some limited insights into the relationships among the *ompL* clusters in *L. interrogans* because a separate cluster with a long diversification period has

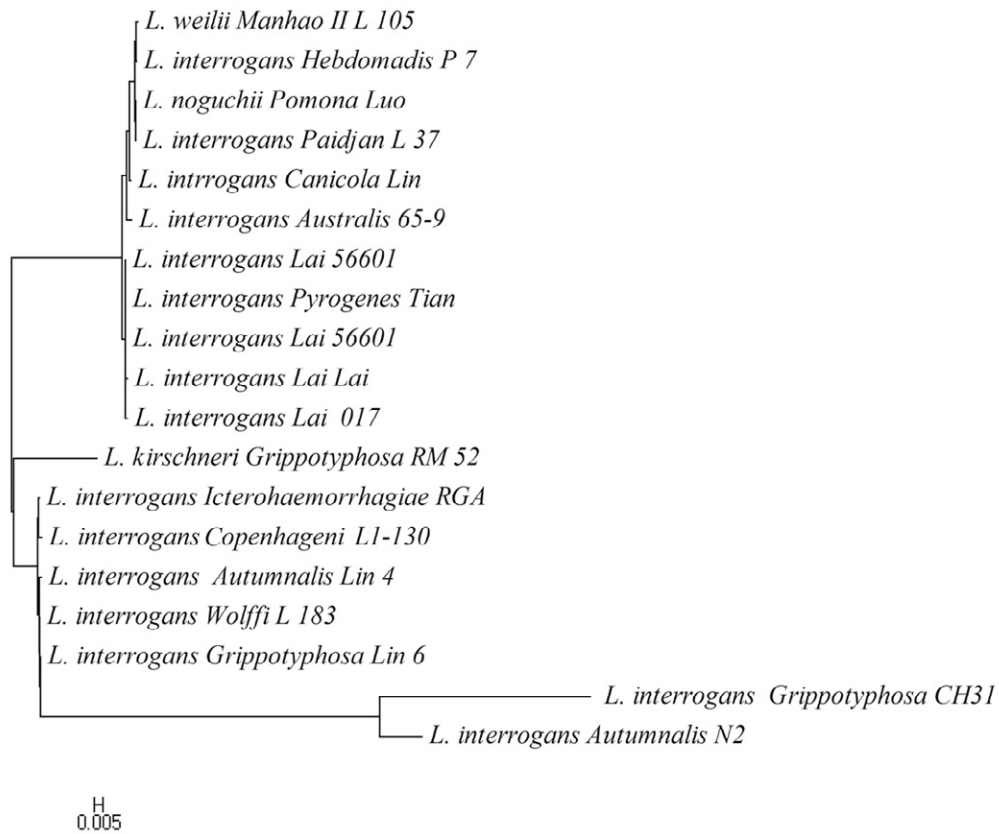


Figure 2 Phylogenetic tree based on *ompL1* nucleotide sequences of *Leptospira* using NJ method.

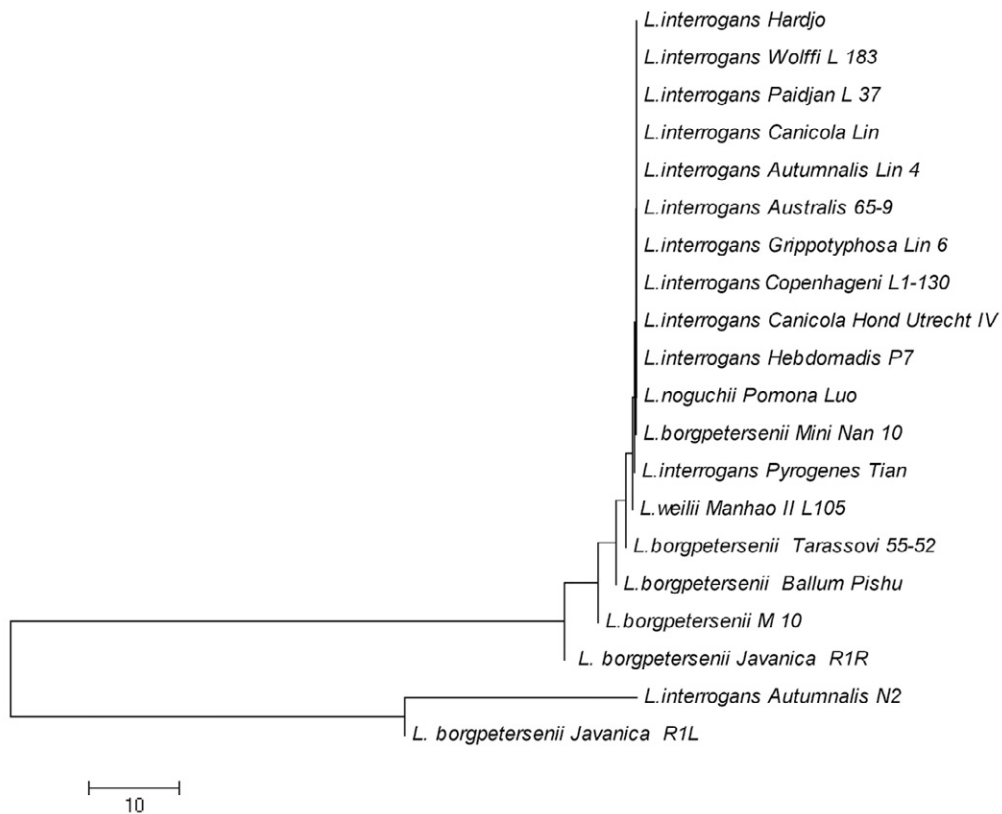


Figure 3 Phylogenetic tree based on *lipL32* nucleotide sequences of *Leptospira* using NJ method.



Figure 4 Phylogenetic tree based on *lipL41* nucleotide sequences of *Leptospira* using NJ method.

been attributed. Nevertheless, we found reasonable levels of relationship within species, but not to the serotypes of this genus.

The thirteen serovars of *Leptospira* in *ompL1* pattern were grouped into three major clusters (Figure 2). In the first cluster, serovars Manhao, Hebdomadis, Pomona, Paidjan, Canicola, Australis, Lai and Pyrogenes formed a monophyletic group and the relatedness exhibited a simple pattern with little distinction. The serovars Grippytyphosa, Icterohaemorrhagiae, Copenhageni, Autumnalis and Wolffi in cluster 2 have some distance with each other, which showed a low similarity with clusters 1 and 3. In cluster 3, serovars Grippytyphosa and Autumnalis clustered together and formed a monophyletic group, but it was closely related to cluster 2. *L. interrogans* with different serovars clustered in a separate clade, suggesting the close evolutionary relationship among them. The NJ tree of *lipL41* gene sequence in Figure 4 showed that serovar Grippytyphosa of *L. interrogans* was distantly related with serovar Autumnalis and then formed a cluster similar to that of *ompL1*. However, *lipL41* of *L. borgpetersenii* group formed a separate cluster consisting Ballum, Javanica and

Tarassovi serovars. As in the *ompL1* tree, the *L. interrogans* group doesn't emerge from the *L. kirschneri* branch. This indicates a greater degree of separation of the *L. interrogans* sequences from the *L. kirschneri* sequences. In Figure 3, it was found that *lipL32* genes of the *L. borgpetersenii* group could not produce a separate cluster as resulted in *ompL1* and *lipL41* trees, but the rest of the other groups almost clustered as *L. interrogans* and *L. borgpetersenii*, *L. weilii*, and *L. noguchii*. This finding indicates that there is a strong sero-specific variation in *lipL41* and *lipL32* gene sequences, which determines the specific antigenic properties of proteins encoded by these genes. Our three gene sequences formed a cluster with members of the *L. kirschneri*, *L. noguchii* and *L. borgpetersenii* species as a separate branch. This is consistent with the finding that the *ompL1*, *lipL32* and *lipL41* sequences of *L. interrogans* strains are not identical to other leptospiral strains presently analyzed in this work.

Among 56 OMP-related genes (deduced amino acids) of different bacterial strains examined, 10 major clusters were formed in the phylogenetic tree as shown in Figure 5, on which our gene sequences have

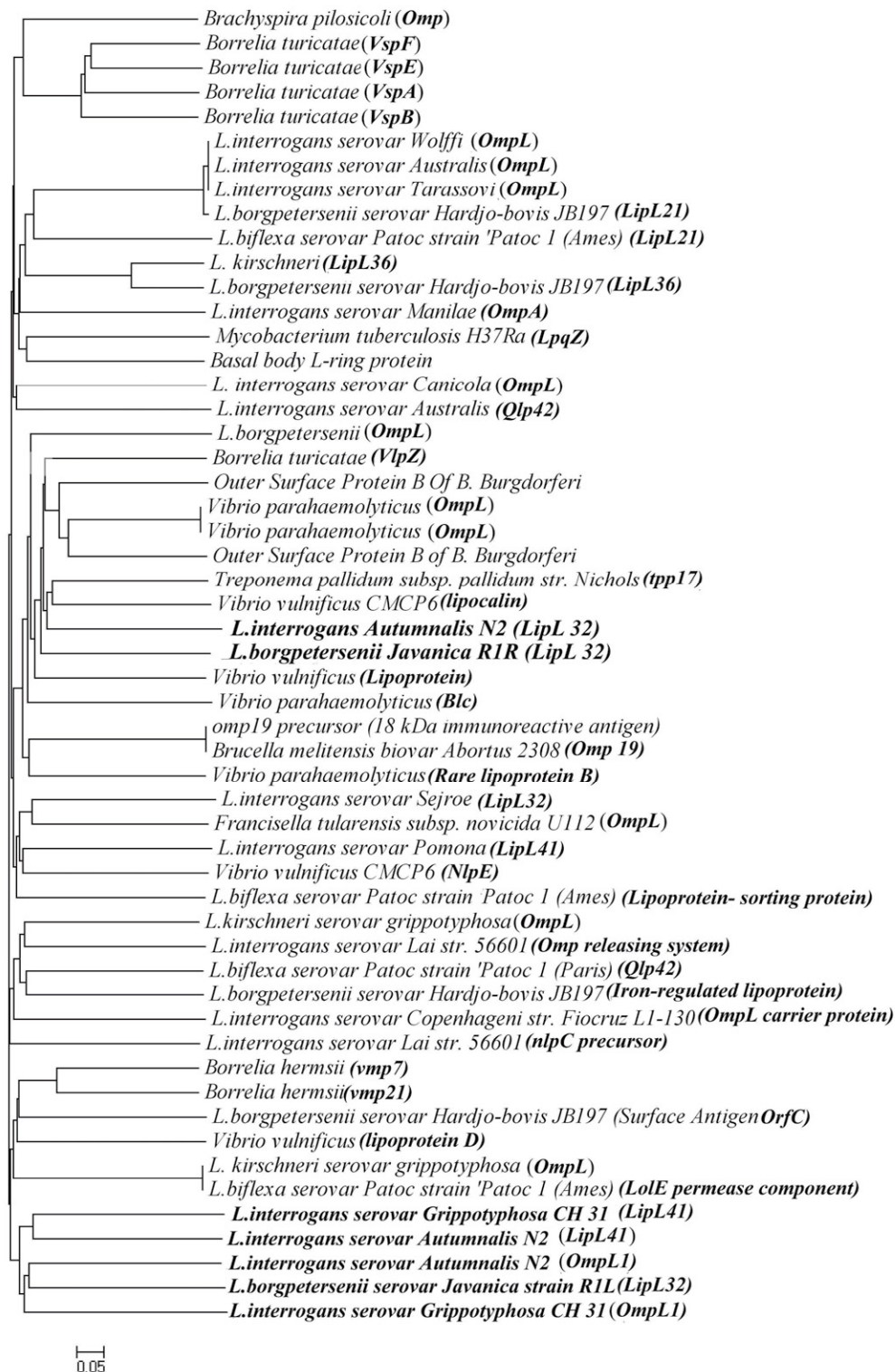


Figure 5 Phylogenetic tree based on deduced nucleotide sequences of OMPs and OMP-related proteins retrieved from different bacterial species using NJ method.

been related together to a great extent. *lipL32* sequence of *L. borgpetersenii* showed similarity to *ompL1* of *L. interrogans*, confirming evolutionary relationship of these strains. Although the relatedness

of the *ompL* gene family was not too diversified, most of them belonged to the same group of organisms but different serovars.

Discussion

Although different strains of the same serovars clustered together, there are some genetic dissimilarities that appear to be accountable for such strain variability in the clinical presentations (19). Similarly, the strong dissimilarities within conserved regions of *ompL* genes were found in *L. interrogans*, which could bring some genetic variations during evolutionary process. Bacterial porin proteins are highly conserved, allowing for the selective movement of hydrophilic solutes through the outer membrane of Gram-negative bacteria (20). Nevertheless, only the difference in sequence similarity could not determine the evolutionary relationship and gene diversity of these *ompL* genes. Some of the homologous characters (orthologs and paralogs) were assigned to reveal how these sequences were diversified from other closely and distantly related organisms. Determination of orthology or paralogy is critically important because paralogous genes often have distinct functional roles in organisms.

The BLAST tool is arguably the most powerful and useful tool in bioinformatics and has been used to functionally annotate millions of genes, providing remarkable insights into biological systems. Although this algorithm is both deceptively simple and remarkably powerful, researchers have recognized that the BLAST algorithm cannot reliably distinguish between orthologous and paralogous genes (21, 22). Therefore, high similarity sequences obtained from BLAST search were subjected to evaluating their phylogeny to find orthologous features. Phylogenetic analyses, on the other hand, easily distinguish orthologs from paralogs given sufficient sampling of related sequences, and these methods have often been used to characterize new functional groups of proteins (23). The sequence identities of *ompL* gene family apart from our *ompL* gene sequences reasonably showed less variation in all aligned sequences; therefore, only closely related leptospiral strains have been used to infer evolutionary significance. We observed that there are strong sequence dissimilarities between *ompL* of *L. interrogans* isolated in this study and *ompL* of the same species used in phylogenetic tree. It may be resulted from key modification of amino acid residues located in antigenic determinant sites of OMPs that are conferring antigenicity of these strains, leading to serovar diversification but not to species evolution. In earlier studies, host environment and geographical regions also contributed for sero-specific (serovar evolution) variation of *L. interrogans* (19, 24). In pathogenic

leptospiral strains, *ompL1* and *lipL41* are highly conserved sequences that are expressed both in cultivated organisms and during infection in mammals (13).

A variation in a particular stretch of amino acids or single amino acid in OMP sequences could bring some specific antigenic variants in these organisms. However, we found only a minor change in *ompL* gene sequences of *L. interrogans* other than the sequences of non-leptospiral strains used in our study. Thus, we assumed that these strains could act as outgroup organisms due to a separate cluster with distinguished evolutionary distance generated. Most of these insertions or deletions are in the variable extracellular regions of the porin regions, which are known to undergo relatively rapid evolutionary change (20). Moreover, there is a lot of evidence supporting that the antigenic variation is resulted due to genetic alternation in highly variable *ompL* genes in *Leptospira*, suggesting the use of an adaptive mode of evolution to escape immune pressures (10).

Changes in the antigenic composition of LPS are also thought to account for serovar diversity (25). Accordingly, serovar diversity of these strains would have been resulted on the basis of diversification process of *ompL* gene family during infection. Sometimes, the entire *lipL41* sequence of *L. interrogans* can also be acquired to *L. borgpetersenii* strain HB10 during the putative transformation event as reported by Haake *et al* (11). Horizontal transfer of DNA appears to involve both entire *lipL41* gene and portions of *ompL1* gene. Three different mosaic patterns of *ompL1* gene recombination were observed, varying from two to four sites of interspecies recombination involving DNA derived from two to three different leptospiral species origins per mosaic pattern (11). The investigators also noted that LipL41 amino acid sequence of *L. interrogans* serovar Pomana is identical (99%) to that of *L. kirschneri* serovar Grippotyphosa (13). In our study, *lipL32* of *L. interrogans* is highly identical to that of *L. borgpetersenii*, suggesting a close phylogenetic relationship between them, which is more distinct than other pathogenic leptospiral strains.

The strains MG569, MG633, D22 and ALC10 of serovar Ratnapura showed close relation to *L. interrogans* associated with pulmonary hemorrhages in Andaman Islands, whereas the strain ALC10, though belonging to the same serovar, showed close relation to *L. kirschneri* and was responsible for hepatorenal failure in south India (19). As the amino acid OmpL1 is highly conserved among pathogenic *Lep-*

toospira species (13), this work also suggested evidence on the unique nature of OmpL1, LipL41 and Lip32 in *L. interrogans* isolated from human urine. Nonetheless, our study on OMPs suggests that implementing an automated phylogenomic approach, combined with gene position analyses and BLAST searches, could significantly enhance the accuracy of gene sequence annotations with special reference to antigenic determinants.

The patterns of substitution in OMPs at both the nucleic acid level and the amino acid level to identify residues may be correlated with serovar-specific tissue tropism or pathogenic potential by virtue of shared evolutionary divergence. The immunological relevance of the increased amino acid sequence variation in OMPs such as *ompL1*, *lipL32* and *lipL41* can now be assessed. The significance of our study is that antigenic and pathogenic characteristics of these strains, notably on serovar-specific ones, are thought to be determined by some sequence variations occurred in *ompL* of *L. interrogans* during evolutionary process. Moreover, such sequence variation will also provide guidance for the development of OMP-specific vaccines for leptospirosis in India and other places.

Materials and Methods

Isolation of leptospiral strains

Human urine samples were processed as per standard procedures from which 1–2 drops of urine was inoculated into Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid medium (Difco, Sparks, USA) in McCartney bottles with a hole cut in the aluminum cap and a rubber lining underneath (26, 27). The clinical samples in this study were collected according to ethical guidelines governed by ethical committee of Regional Medical Research Centre (ICMR), Port Blair and perception of individual patients. The rat

(*Rattus norvegicus*) kidney was aseptically dislocated from field rats and a piece of fleshy tissue was immediately inoculated in EMJH medium (28).

Growth and maintenance of leptospiral strains

The isolated leptospiral strains were cultivated in EMJH medium with additional supplementation of 0.2% agarose (Sigma, USA), 1% bovine serum albumin (Sigma), 2% rabbit serum, 0.1% sodium pyruvate, and 100 µg/mL 5-fluorouracil (as a selective agent). Every sample in triplicates was inoculated in tubes containing the above medium, incubated at 30°C in dark and the presence of leptospire was examined at weekly intervals using a dark field microscope. The positive growth of these strains was subcultured in vials having fresh EMJH medium and further incubated at 30°C for 7 days. All strains were identified up to serovar level and maintained in the leptospiral repository in RMRC Port Blair with a periodic regeneration in EMJH medium. The N2 strain of urine isolate, CH31 strain of human blood isolate, R1R and R1L strains of rat isolates (unpublished) were obtained and utilized for this study (24).

DNA extraction, amplification and sequencing

DNA was extracted from seven-day-old broth cultures of *Leptospira* according to Boom *et al* (29). The genes encoding for OmpL1, LipL41 and LipL32 proteins were amplified from the purified DNA by using PCR primers as listed in Table 4. Briefly, each 50 µL PCR reaction mixture contained approximately 50 ng of purified DNA, 0.1 µM primer, 250 µM of each dNTP (Fermentas, USA), 25 mM MgCl₂, 0.5 U of Taq DNA Polymerase (Fermentas), 10 mM Tris-HCl (pH 9) and 50 mM KCl. Amplification was performed in thermal cycler (Eppendorf, Germany) at 94°C for 5

Table 4 PCR primer sequences for amplifying *ompL* genes studied in this work

Primer*	Oligonucleotide sequence
<i>ompL1</i> F	5'-TTGATTGAATTCTTAGAGTTCGTGTTTATA-3'
<i>ompL1</i> R	5'-AAGGAGAAGCTTATGATCCGTAACATAAGT-3'
<i>lipL32</i> F	5'-TTACCGCTCGAGGTGCTTTTCGGTGGTCTGC-3'
<i>lipL32</i> R	5'-TGTTAACCCGGGTTACTTAGTCGCGTCAGA-3'
<i>lipL41</i> F	5'-AAAGGACTCGAGTTACTTTGCGTTGCTTTC-3'
<i>lipL41</i> R	5'-TGTTACCCATGGGGAGAAAATTATCTTCTCT-3'

*F: forward primer; R: reverse primer.

min, followed by 40 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 60 s, with a final single extension of 72°C for 7 min. The annealing temperature of 51°C (*lipL32*), 58°C (*lipL41*) and 60°C (*ompL1*) were used. Amplified products were characterized by electrophoresis of 5 µL of each reaction on a 1.2% agarose gel for 30 min at 85 V. Sequencing reaction products were purified with magnetic carboxylate beads (Agencourt Bioscience, Beverly, USA) and sequenced on an AB 3100 sequencer (Applied Biosystems, USA).

Phylogenetic analysis

The highly identical sequences for *ompL1*, *lipL32* and *lipL41* used in this study were retrieved from NCBI database using BLASTn and PSI-BLAST tools with default parameters (30). Sequences with more significant identity were aligned with ClustalW algorithm implemented in MEGA 4.0 (31) using Smith-Waterman substitution matrix and trimmed to consensus, and NJ and ME algorithms were used to construct phylogenetic trees using MEGA 4.0 with 1,000 bootstraps at uniform divergence rates with Jukes and Cantor evolutionary model and 0.25 gamma distribution factor. Gaps in the aligned sequences were replaced by Ns in BioEdit 7.0.4.1 (32). Maximum likelihood (ML) with molecular clock v3.6a2.1 tree was constructed with DNAMLK 3.5c program at a constant rate variation with substitution rate and 2 transition/transversion ratio. Posterior probability and conserved regions among closely related sequences were carried out by MEGA 4.0 and BioEdit 7.0.4.1, respectively.

GenBank accession numbers

Nucleotide sequences have been deposited in the GenBank of NCBI database with the accession numbers EU091293–EU091296 and EU293441–EU293443.

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Authors' contributions

KV carried out experiments throughout this study and prepared this manuscript. KN conceived the idea of using this approach, supervised the project and co-wrote this manuscript. PC contributed in phylogenetic analysis and assisted with manuscript preparation. SGP and JS supported to isolate the strains and provided lab facilities. SS and PV helped to identify the strains. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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