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Biotehnology

Cloning, Phylogenetic Analysis and Expression of Recombinant LipL41, Loa22 and LipL21 Proteins from *Leptospira interrogans*

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Abstract

Leptospirosis, a worldwide anthropozoonotic infection with multisystemic involvement, is emerging in North India. These days, focus is to develop recombinant outer membrane protein based diagnostic tests. In this study, three genes *lipL41* (1088 bp), *loa22* (608bp) and *lipL21* (581bp) of *Leptospira interrogans* were cloned and sequenced. Multiple sequence alignment and phylogenetic analysis revealed that *loa22* and *lipL21*gene sequences of *L. interrogans* serovars Grippotyphosa and Canicola respectively were conserved in nature but *lipL41* gene sequence of *L. interrogans* serovars Grippotyphosa showed variation in nucleotide sequence which contributes to serovar evolution within species. For protein expression truncated *lipL41* (1028 bp), *loa22* (548bp) and *lipL21* (472bp) genes were amplified, cloned and expressed in prokaryotic expression system and His-tagged ~45kDa (*lipL41* gene), ~28kDa (*loa22* gene) and ~17kDa (*lipL21* gene) proteins were purified by nickle-nitriloacetic acid (Ni-NTA) affinity chromatography. Purified proteins were confirmed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. For immunological characterization, total four doses of recombinant proteins were injected subcutaneously into Swiss-albino mice at 50µg quantity along with Freund's adjuvant and after 21 days immunogenicity of expressed proteins was tested by blotting using mice raised serum.

Highlights

- Amplification of *lipL41* (1088 bp), *loa22* (608bp) and *lipL21* (581 bp) *Leptospira interrogans* were cloned and sequenced.
- *loa22* and *lipL21* genes sequences were conserved in nature but *lipL41* gene sequence showed variation in nucleotide sequence resulting evolution within species.

Keywords: *Leptospira*, Outer membrane protein, Cloning, Expression, Blotting

Leptospirosis is a worldwide zoonotic disease, also known as Weil's disease. It is caused by thin spiral shaped organism which belongs to the family Leptospiraceae, order Spirochaetales and genus *Leptospira* (Faine *et al.*, 1999). *Leptospira* genus includes free-living non pathogenic species as well as pathogenic species which can infect both humans and animals. Leptospirosis is a major re-emerging public health problem in developing countries (Levett, 2001; Bharti *et al.*, 2003; McBride *et al.*, 2005). Mostly the outbreaks have been observed in

flooded areas because of water contamination (Zaki and Sheih, 1996; WHO, 2000; Sehgal *et al.*, 2001). Outbreaks of leptospirosis have been reported from India, Brazil, China, Iran and Sri Lanka (Hartskeerl, 2005; Wang *et al.*, 2007; Jamshidi *et al.*, 2008; Pappas *et al.*, 2008; Sethi *et al.*, 2010). This disease is a serious problem in several south Indian states but from the last few years cases have been appearing in large number in several north Indian states including Delhi, Punjab and Haryana (Pooja *et al.*, 2001; Chaudhry *et al.*, 2002).

There are different diagnostic methods available like Dark-field microscopy, Phase contrast microscopy, Silver staining, Polymerase Chain Reaction (PCR), Isolation of leptospires, Microscopic Agglutination Test (MAT), Lepto-Dipstick, Enzyme linked immunosorbent assay (ELISA) etc. Among these ELISA has been found to be simple, safe, easy to automate and a very suitable assay for the examination of large number of sera samples. As the number of cases has been increasing, steps are being taken to diagnose the disease as early as possible. Multi serovar ELISA assays based on recombinant LipL41, LipL21, LipL32 and OmpL1 proteins have been developed for serodiagnosis of Leptospirosis (Theodoridis *et al.*, 2005; Zhang *et al.*, 2005; Chalayon *et al.*, 2011) as it replaces the handling of highly pathogenic *Leptospira*.

In *Leptospira* the most abundant class of lipoproteins comprise LipL32, LipL36, LipL41, LipL48, LipL21, temperature- regulated Qlp42; transmembrane protein OmpL1; and peripheral membrane protein LipL45 (Matsunaga *et al.*, 2002 a). LipL32, LipL41, and OmpL1 are major antigens to induce the humoral immune response to leptospirosis because these proteins are at the interface between the pathogen and the mammalian host immune responses (Flannery *et al.*, 2001; Guerreiro *et al.*, 2001 a). The immunodominancy of 41 kDa protein has been reported (Shang *et al.*, 1996) in serovar Grippotyphosa. Surface membrane lipoproteins LipL45 (Matsunaga *et al.*, 2002 b) and LipL21 (Cullen *et al.*, 2003) are produced during infection and conserved among pathogenic *Leptospira* species. Loa22 is a lipoprotein of *Leptospira interrogans* having Outer membrane protein A (OmpA) domain in the C-terminus. Loa22 has been identified to be present among pathogenic leptospires but not among non-pathogenic leptospires, suggesting the possible involvement of this protein in virulence (Zhang *et al.*, 2010). In other bacteria, OmpA acts as a multifunctional protein involved in cell adhesion, tissue invasion and induction of the immune response (Torres *et al.*, 2006). In view of the above mentioned facts, objectives of the present study were to express LipL41, Loa22 and LipL21 proteins from indigenous strains in recombinant form and to immunologically characterize these in experimental animal model (mice).

Materials and Methods

Cell Lysate Preparation

Leptospira interrogans serovars Grippotyphosa and Canicola were procured from Regional Medical Research Centre, Indian Council of Medical Research, Port Blair, Andaman & Nicobar Islands (India). *Leptospira* cultures were maintained on Leptospira Medium (LM) supplemented with Leptospira Enrichment (Himedia, Mumbai) at 29°C in BOD incubator. After two weeks genomic DNA was extracted by using hot cold lysis method. 1ml of culture was boiled at 100°C for 10 min then chilled at 0°C for 5 min; after that centrifugation was done at 10,000 rpm for 10 min. Supernatant was used as template for PCR.

Cloning and Sequencing

Sequences for *lipL41, loa22* and *lipL21* genes were downloaded from NCBI's GenBank database and primers were designed after multiple sequence alignment of the available sequences by ClustalW (Table 1). For directional cloning *NcoI* and *XhoI* restriction endonuclease (RE) sites were included in the forward and reverse primers respectively. *lipL41, loa22* genes were amplified from serovar Grippotyphosa DNA template while *lipL21* gene was amplified from serovar Canicola DNA template. For polymerase chain reaction (PCR) 25µl reaction mixture containing 1X PCR buffer, 2.5mM magnesium chloride ($MgCl_2$), 200µM deoxyribonucleotide triphosphate (dNTP) mix, 0.4µM of each of forward and reverse primer, 2.5U of Taq DNA polymerase (Fermentas Inc., Maryland USA) and ~50ng of template DNA was prepared. Cycling conditions were standardized using Master Cycler Ep Gradient S (Eppendorf, Germany) including one cycle of

Table 1. Primers designed for amplification of lipL41, loa22 and lipL21 genes

Gene	Primer sequence (5'- 3')	Restriction enzyme sites	Length of amplicon (bp)
lipL41 gene	F: cgcccatggccatgagaaaa R: cgcctcgagttactttgcgt	*Nco*I *Xho*I	1088
tru *lipL41* gene	TRU F: cgcccatggccgcagctaca TRU R: cgcctcgagttactttgcgt	*Nco*I *Xho*I	1028
loa22 gene	F: cgcccatggccatggtcaaaa R: cgcctcgagttattgttgtgg	*Nco*I *Xho*I	608
tru *loa22* gene	TRU F: cgcccatggcctgctcctctg TRU R: cgcctcgagttattgttgtgg	*Nco*I *Xho*I	548
lipL21 gene	F: cgcccatggccatgatcaa R: cgcctcgagttattgtttgg	*Nco*I *Xho*I	581
tru *lipL21* gene	TRU F: aaccatggacacaggacaaaaagacg TRU R: aactcgagacgagagcatccttacca	*Nco*I *Xho*I	472

Underlined are restriction enzyme sites

initial denaturation (94˚C/5 min) followed by 35 cycles each of denaturation (94˚C/1 min), annealing (60˚C/1 min for *lipL41*gene, 50˚C/1min for *loa22* gene and 55˚C/1 min for *lipL21* gene) and extension (72˚C/1 min) followed by final extension (72˚C/10 min). The PCR products were analyzed by electrophoresis on 1.5% agarose gel prepared in 0.5X tris/borate/EDTA (TBE) buffer and visualized on ChemiDoc XRS gel documentation system (Biorad, USA). For cloning *lipL41*, *loa22* and *lipL21* genes were amplified in bulk and after gel extraction (Qiagen, USA) PCR products were ligated with pGEMTEasy^{TM} cloning vector at 4˚C for overnight (Promega, USA). Ligated products were transformed into DH5α (*E.coli*) cells by calcium chloride ($CaCl_2$) method. Randomly, six white colonies were picked by blue-white screening for each gene and plasmids were isolated by alkaline lysis method using standard plasmid extraction protocol. One positive clone, for each gene, confirmed by restriction digestion (*EcoRI* (Fermentas Inc., Maryland USA)) was sent for commercial sequencing to DNA Sequencing Facility, University of Delhi (South Campus), Delhi, India.

Phylogenetic Analysis

After sequencing the obtained complete cds of the *lipL41, loa22* genes in *L. interrogans* serovar Grippotyphosa and of *lipL21* gene in *L. interrogans* serovar Canicola were subjected to BLAST search (www.ncbi.nlm.gov/Blast) using BLASTn program of NCBI (Altschul *et al.*, 1997). In the search result of BLASTn, the sequences showing higher homology with *lipL41, loa22* and *lipL21* gene sequences were aligned using ClustalW2 multiple sequence alignment program of European Bioinformatics Institute and European Molecular Biology Lab database (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The same homologous sequences were used to generate phylogenetic tree using Tree View program of NCBI (BLAST pair wise alignment) with fast minimum evolution method.

Expression of Recombinant Proteins

For amplification of truncated *lipL41*, *loa22* and *lipL21* genes primers were designed after multiple sequence alignment of the available sequences on NCBI database (Table 1). For directional cloning of the truncated genes

NcoI and *XhoI* restriction endonuclease (RE) sites were included in the forward and reverse primers respectively. Putative signal sequences were removed at the 5' end of the coding sequences (not included in the forward primer) of the genes. The PCR assay was standardized for selected three genes in 25µl reaction mixture to get a single band. The composition of PCR mixture was 1X PCR buffer, 2.5mM MgCl2, 200µM dNTP mix, 0.4µM of each of forward and reverse primers, 2.5U of Taq DNA polymerase (Fermentas Inc., Maryland USA) and ~50ng of template DNA. Cycling conditions were standardized using Master Cycler Ep Gradient S (Eppendorf, Germany) having one cycle of initial denaturation (94˚C/5 min) followed by 35 cycles each of denaturation (94˚C/1 min), annealing (60˚C/1 min for tru *lipL41gene*, 55˚C/1 min for tru *loa22* gene and 60˚C/1 min for tru *lipL21* gene) and extension (72˚C/1 min) followed by final extension (72˚C/10 min). The PCR products were analyzed by electrophoresis on 1.5% agarose gel prepared in 0.5X TBE buffer and visualized on ChemiDoc XRS gel documentation system (Biorad, USA).

Truncated *lipL41*, *loa22* and *lipL21* genes were amplified in bulk and gel was extracted using Qiagen gel extraction kit (Qiagen, USA). pProExHT (b) prokaryotic expression vector (Invitrogen, USA) was used to ligate amplicons of *lipL41*, *loa22* genes and pProExHT (a) prokaryotic expression vector was used for ligation of *lipL21* PCR product. Eluted PCR products and pProExHT prokaryotic expression vectors (Invitrogen, USA) were double digested with *NcoI* and *XhoI* restriction enzymes and ligated at 14˚C for overnight. Ligated products were transformed in DH5α (E.coli) competent cells by $CaCl_2$ method. Plasmids were isolated by alkaline lysis method from randomly picked six white colonies for each gene. After that clones were confirmed by restriction double digestion of the isolated plasmids with *NcoI* and *XhoI* (Fermentas Inc., Maryland USA) restriction enzymes. Results were analyzed on 1.5% agarose gel prepared in 0.5X TBE buffer and visualized on ChemiDoc XRS gel documentation system (Biorad, USA).

Induction of Expression

For expression study of recombinant LipL41, Loa22 and LipL21 proteins, one positive clone for each gene was grown in LB broth containing ampicillin (100µg/ml) to the level of 0.3 OD (at 600nm) and induced by adding 0.6 mM isopropyl β–D thiogalactopyronoside (IPTG). 2.0 ml sample was collected at 0 hr then after 6 hrs and analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Expressed recombinant proteins were purified under denaturing condition by nickel-nitriloacetic acid (Ni-NTA) affinity chromatography (Qiagen, USA). Lysis buffer (pH8.0), washing buffer (pH 6.3), and elution buffer (pH 4.5) were prepared according to standard composition and 5mM imidazole was added in washing buffer for LipL41 and Loa22 protein purification. Collected purified proteins were again confirmed by SDS-PAGE analysis. Small sized contaminant proteins (if any) and salts (urea) were removed from purified proteins by dialysis against 1X PBS using a dialysis tubing of 6kDa cut off value.

Western Blotting

For confirmation of his-tagged purified proteins by western blotting, proteins were separated on SDS-PAGE. Following the separation, proteins were electro blotted on to nitrocellulose membrane (NCM) using a semidry blotter (Atto, Japan). Three times washing was given to NCM with tris-buffered saline (TBS) each for 10 min. Blocking was done for overnight at 4˚C with 3% bovine serum albumin (BSA) (SRL, India) followed by three times washing with tris-buffered saline-tween 20 (TBS-T). Nickel-nitrilotriacetic acid-horseradish peroxidase (Ni-NTA HRP) conjugate (Qiagen, USA) was diluted in 1:1000 using TBS-tween 20 buffer and NCM was soaked in to it for 1hr at 37˚C. After washing for three times with TBS-T buffer, membrane was soaked in staining solution. Staining solution having 1.25ml of 2M Tris (pH 7.5), 25mg diaminobenzidine (DAB), 150µl of 8% nickel chloride and 30µl of 30% hydrogen peroxide (H2O2) was used. Development of color was observed and reaction was stopped by rinsing the membrane in distilled water.

Immunoblot with Mice Serum

For immunological characterization, recombinant proteins were injected into Swiss-albino mice (n=4 for each protein) at 50µg quantity along with Freund's

adjuvant. Each dialyzed rLipL41, rLoa22 and rLipL21 protein was mixed in equal quantity with Freund's complete adjuvant to make final concentration of 50µg of protein. On first day each mice was injected with a dose of 250µl subcutaneously of Freund's complete adjuvant preparation. For second, third and fourth booster doses, both recombinant proteins were mixed with Freund's incomplete adjuvant to make final concentration of 50µg of protein. 250µl of the preparation was then injected subcutaneously into each mice at 7 day interval after first dose. After 21 days blood samples were collected from the mice tail and serum was separated from clotted blood. Blotting was performed as described earlier for Ni-NTA western blotting using mice serum as primary antibody (1:500) and rabbit anti-mouse IgG HRP conjugate as secondary antibody (1:500).

Results and Discussion

Cloning of lipL41, loa22 and lipL21 genes

The growth of L. interrogans serovars Grippotyphosa and Canicola appeared within 10-12 days in the form of turbidity. Genomic DNA was extracted using hot cold lysis method. *lipL41*, *loa22* genes were amplified from serovar Grippotyphosa and *lipL21* gene was amplified from serovar Canicola. PCR amplification of *lipL41*, *loa22* and *lipL21* genes resulted in an amplicon of 1088 bp (Fig 1a), 608 bp (Fig 1b) and 581bp (Fig 1c) respectively. Further PCR products ligated with pGEMTEasy^{TM} cloning vector, after transformation in DH5α host cell resulted in blue-white colonies. RE digestion of plasmids with *EcoRI* restriction enzyme resulted in release of specific size inserts of 1088 bp for *lipL41* gene, 608 bp for *loa22* gene and of 581bp for *lipL21* gene.

Sequencing and phylogenetic analysis

Three genes *lipL41*, *loa22* and *lipL21* of Leptospira were amplified, cloned, sequenced and after submission to NCBI's GenBank database following accession numbers for three genes were obtained: lipL41 gene- JQ690557; loa22 gene- KC311551; lipL21 gene- JQ228529. BLASTn search result for lipL41 gene of serovar Grippotyphosa revealed a high degree of homology ranging 96% to 89% with *L. interrogans* serovar Canicola, Hardjo, Paidjan, Wolffi, Hebdomadis, Pyrogenes, Copenhageni and Javanica. For *loa22* gene of serovar Grippotyphosa showed 97% to 86% homology with *L. interrogans* serovar Copenhageni, Lai, Manilae and *L. borgpetersenii* Hardjo-bovis. Similarily for *lipL21* gene of serovar Canicola showed 99% homology to 92% homology with *L. interrogans* serovar Canicola, Grippotyphosa, Hebdomadis, Autumnalis, Tarassovi, Paidjan, Wolffi, Lai, Australis. The search results for three genes which

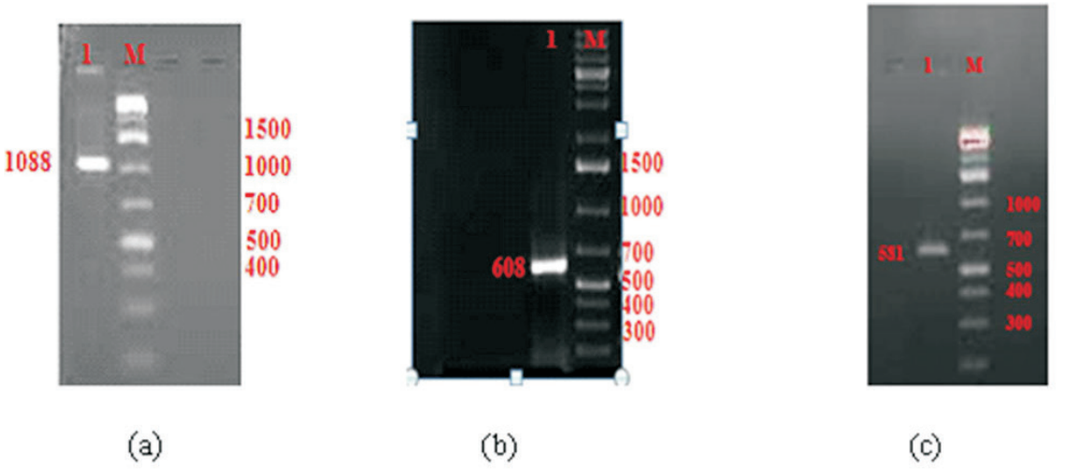

Fig. 1. (A) PCR amplicon of lipL41 gene from L. interrogans serovar Grippotyphosa, (B) PCR amplicon of loa22 gene from serovar Grippotyphosa, (C) PCR amplicon of lipL21 gene from serovar Canicola. Lane 1: Amplicons in bp, Lane M: GeneRulerTM 1kb plus DNA ladder (Fermentas, USA).

Fig. 2. Phylogenetic tree presentation with fast minimum evolution method for lipL41 gene of Leptospira interrogans serovar Grippotyphosa

revealed high percentage of homology in BLASTn were selected for multiple sequence alignment and phylogenetic tree presentation. Serovar Grippotyphosa for *lipL41* gene revealed a difference of near about 49 nucleotides in multiple sequence alignment with subject sequences. But multiple sequence alignment of *lipL21* gene sequence from serovar Canicola showed very few differences in nucleotides with subject sequences. Similarly *loa22* gene from Serovar Grippotyphosa revealed high similarity with nucleotide sequences of serovars Lai, Copenhageni and Manilae but a difference of near about 65 nucleotides was observed with *L. borgpetersenii* serovar Hardjo-bovis. According to phylogenetic tree (Fig. 2) *lipL41* gene of serovar Grippotyphosa belongs to two groups. In group I serovar Canicola showed the close relationship with Hebdomadis and in group II Hardjo, Paidjan, Grippotyphosa serovars were diverged from Pyrogenes, Australis and Wolffi serovars. In *loa22* gene phylogenetic tree (Fig 3) serovar Grippotyphosa was in close relationship with Lai, Copenhageni and Manilae serovars that were diverged from *L. borgpetersenii* serovar Hardjo-bovis. Similarily in phylogenetic tree (Fig 4) for *lipL21* gene serovar Canicola revealed close relationship with serovar Grippotyphosa but these were diverged from the other group that included Hebdomadis, Autumnalis, Tarassovi, Paidjan, Djasiman and Icterohaemorrhagiae and Australis serovars.

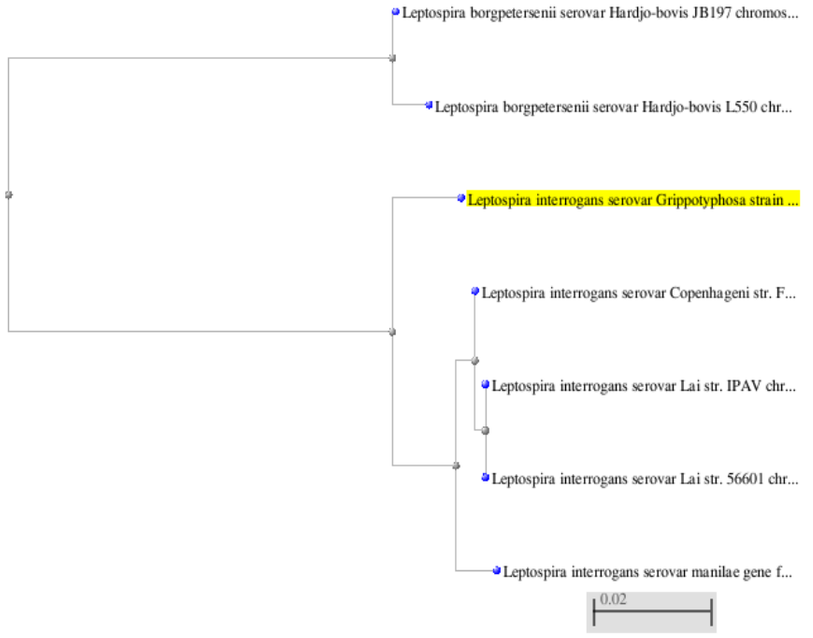

Fig. 3. Phylogenetic tree presentation with fast minimum evolution method for loa22 gene of Leptospira interrogans serovar Grippotyphosa

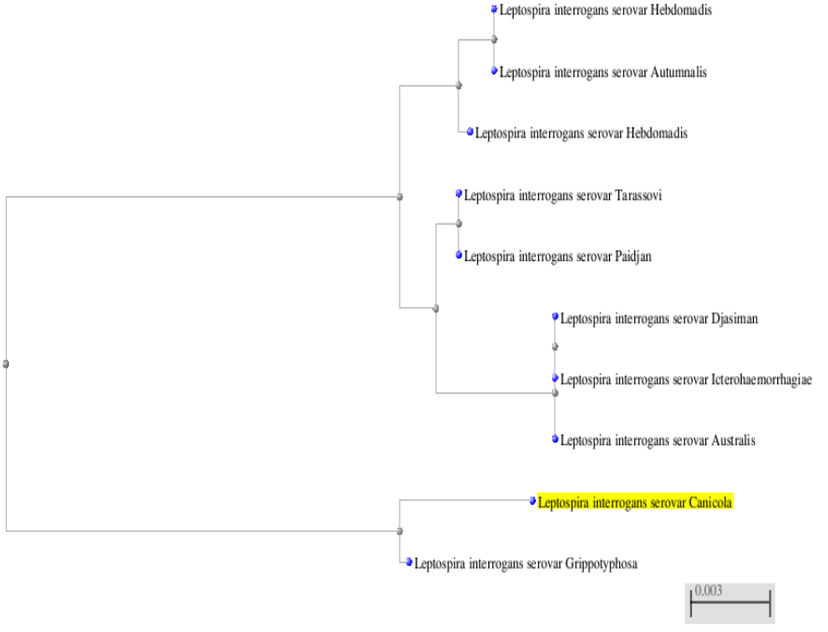

Fig. 4. Phylogenetic tree presentation with fast minimum evolution method for lipL21 gene of Leptospira interrogans serovar Canicola

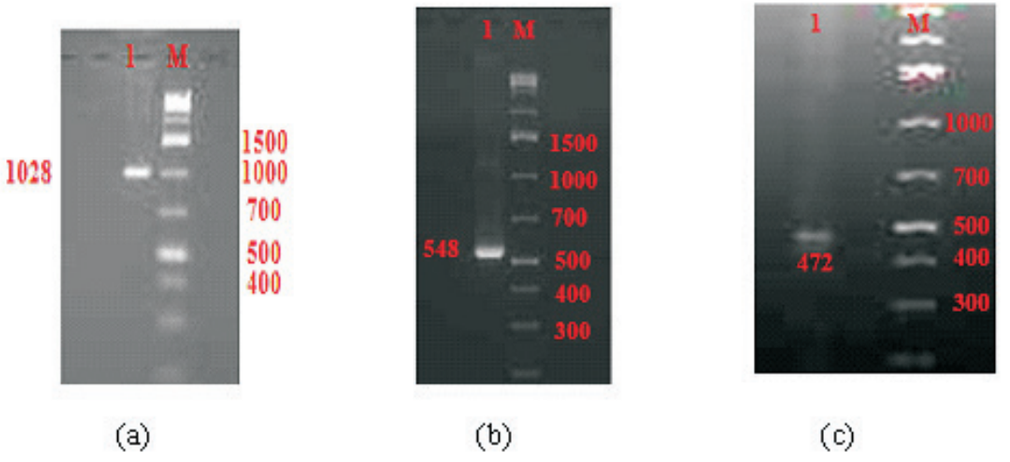

Fig. 5. (a) PCR amplicon of lipL41 tru gene from L. interrogans serovar Grippotyphosa,
(b) PCR amplicon of loa22 tru gene from serovar Grippotyphosa,
(c) PCR amplicon of lipL21 tru gene from serovar Canicola.
Lane 1. Amplicons in bp, Lane M. GeneRulerTM 1kb plus DNA ladder (Fermentas, USA).

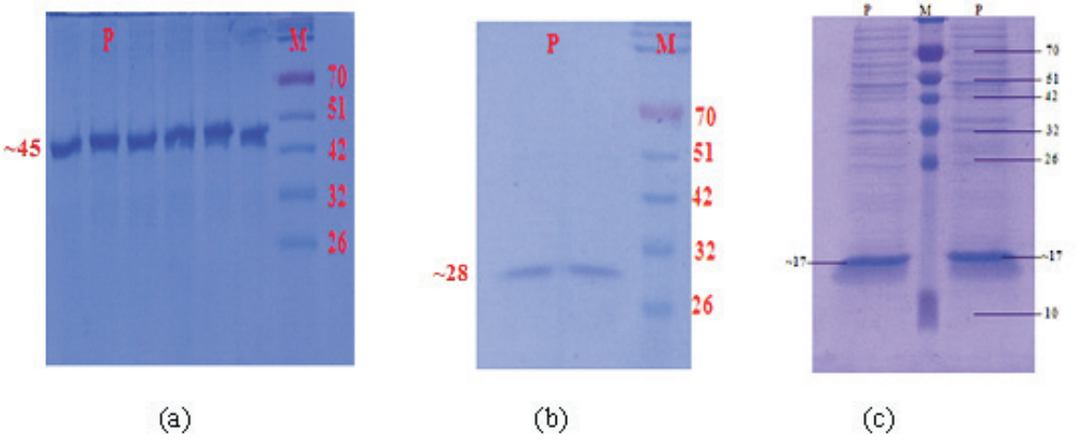

Fig. 6. Purified recombinant proteins by Ni-NTA affinity chromatography.
(a) Lipl41protein (b) Loa22 protein (c) LipL21 protein.
Lane P. Protein in kDa, Lane M. Multicolour broad range protein ladder (Puregene, Genetix Biotech, India).

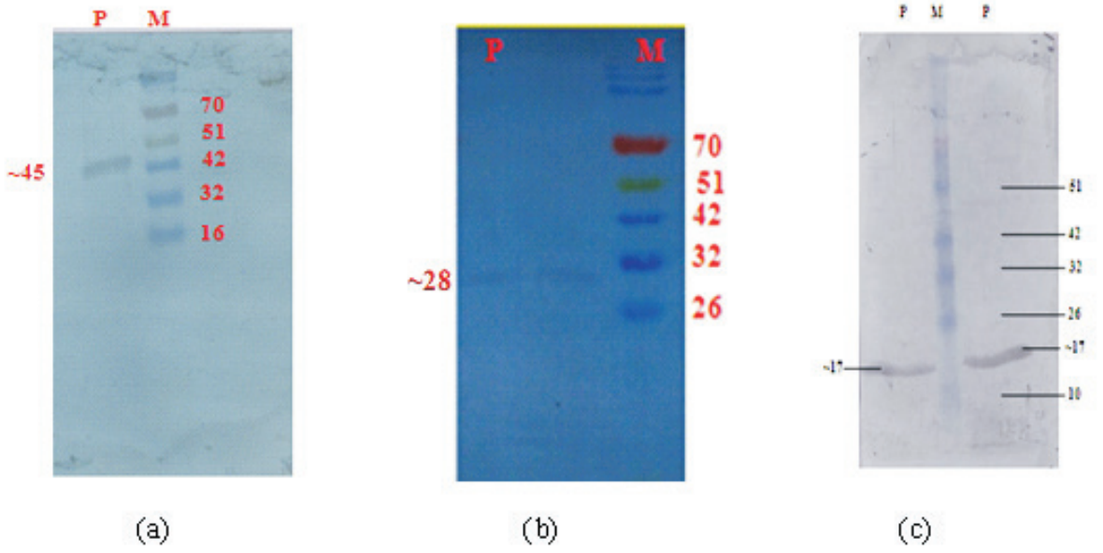

Fig. 7. Western blotting of recombinant proteins using Ni-NTA HRP conjugate. A. Lipl41protein B. Loa22 protein C. LipL21 protein. Lane P: Protein in kDa, Lane M: Multicolour broad range protein ladder (Puregene, Genetix Biotech, India).

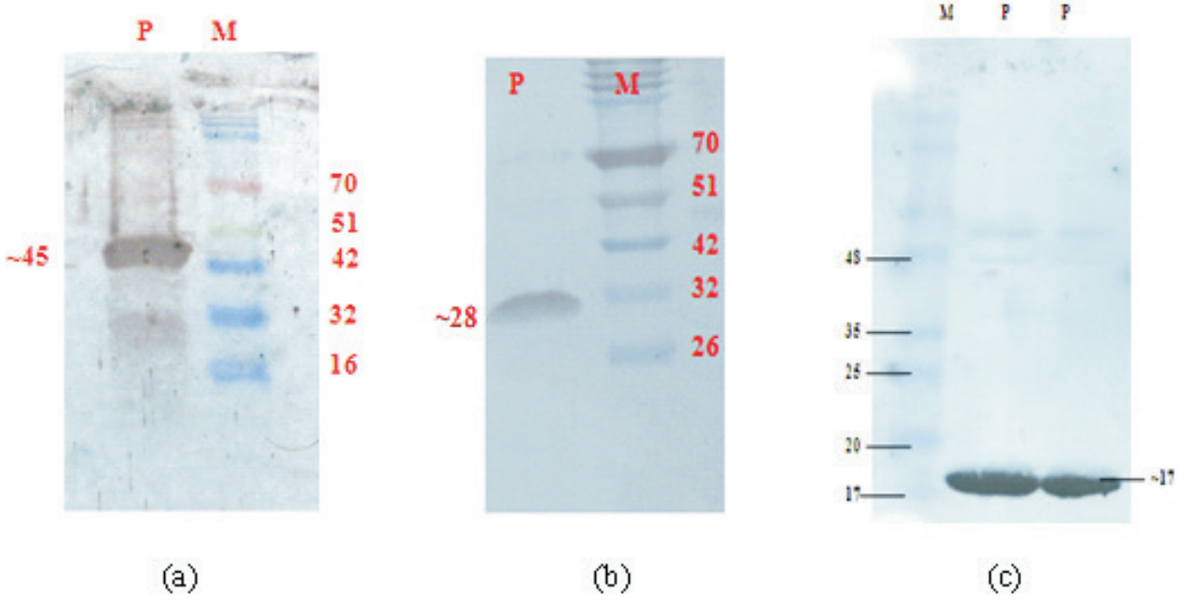

Fig. 8. Immunological characterization of recombinant proteins using mice hyperimmune serum.
(a) Lipl41protein, (b) Loa22 protein, (c) LipL21 protein.
Lane P. Protein in kDa, Lane M. Multicolour broad range protein ladder (Puregene, Genetix Biotech, India) in fig a & b; Broad range protein ladder (BR Biochem Life Sciences, India) in fig c.

Expression of recombinant proteins

PCR amplicons of 1028 bp (Fig 5a), 548 bp (Fig 5b) and 472bp (Fig 5c) size were obtained for *lipL41*, *loa22* and *lipL21* truncated genes respectively. After transformation truncated gene products resulted in the development of numerous white colonies. Restriction double digestion of the isolated plasmids from clones with *NcoI* and *XhoI* enzymes resulted in release of specific size inserts of 1028 bp, 548 bp and 472bp. SDS-PAGE analysis of induced clones resulted in a thick band of ~45 kDa for LipL41 protein, ~28 kDa for Loa22 protein and ~17 kDa for LipL21 protein as compared to un-induced control.

Characterization of recombinant proteins

Purification of recombinant proteins by Ni-NTA affinity chromatography resulted in a specific ~45 kDa band for LipL41 protein (Fig 6a), ~28 kDa for Loa22 protein (Fig 6b) and ~17 kDa for LipL21 protein (Fig 6c). After dialysis western blotting of each recombinant protein with Ni-NTA HRP conjugate resulted in color band at locations of ~45 kDa (Fig 7a), ~28 kDa (Fig 7b) and ~17 kDa (Fig 7c) corresponding to pre-stained protein ladder. In immuno-blotting, using mice serum as primary antibody and rabbit anti-mouse IgG HRP conjugate as secondary antibody, bands at a location of ~45 kDa (Fig 8a), ~28 kDa (Fig 8b) and ~17 kDa (Fig 8c) were obtained.

The genes from *L. interrogans* serovar Grippotyphosa (*lipL41*, *loa22*) and serovar Canicola (*lipL21*) were cloned, sequenced, analyzed and then expressed in recombinant form for immunological characterization in mice that may be further used in serodiagnosis and as vaccine candidate for leptospirosis. After sequencing, in multiple sequence alignment analysis few variations were observed in *loa22* nucleotide sequences of *L. interrogans* serovars Grippotyphosa, Lai, Copenhageni and Manilae which showed that *loa22* gene was highly conserved throughout the evolutionary path but more variations were observed in *L. borgpetersenii* serovar Hardjo-bovis gene sequence. Similarly phylogenetic tree presentation revealed that Leptospira species *L. interrogans* and *L. borgpetersenii* both are pathogenic but in evolutionary path these were diverged from each other. *lipL21* gene sequence was highly conserved in *L. interrogans* serovars Grippotyphosa and Canicola showing few variations in nucleotide sequences thus indicating their common ancestral origin with high percentile of homology. OMP genes encoding LipL21, LipL32 and OmpL1 proteins have been reported to be highly conserved among pathogenic Leptospira species *L. interrogans*, *L. borgpetersenii* and *L. weilii* (Wu *et al.,* 1996 ; Guerreiro *et al.,* 2001b). The nucleotide sequence of the Canicola *lipL21* gene showed high percentile of homology with serovars Pomona, Lai, Bratislava, Grippotyphosa and Mozdok (Cheema *et al.,* 2007). According to *loa22* and *lipL21* gene sequences analysis, serovars Grippotyphosa and Canicola were very closely related but *lipL41* gene sequences from both the serovars revealed variation of near about 49 nucleotides. Dissimilarities between *ompL1* gene sequences of *L. interrogans* serovars confer that variations sometimes lead to serovar diversification but not to species evolution as it depends on host environment and geographical regions (Roy *et al.,* 2003; Natarajaseenivasan *et al.,* 2005). Similarly variations in *lipL41* gene sequences of *L. interrogans* serovars indicated the progress for serovar evolution that led to generation of new strains but not to new species. Cloned genes were amplified in truncated form to express proteins that were purified as his-tagged recombinant proteins and sometimes his-tag lead to increase in molecular weight of proteins. Immuno-blotting study showed antiserums raised in mice against *rLipL41*, *rLoa22* and *rLipL21* proteins successfully reacted with proteins and revealed that they mimic the role of natural surface exposed LipL41, Loa22 and *LipL21* antigens of Leptospira. *Loa22*, *OmpL1* and *LipL41* proteins were similarly cloned, expressed and characterized by immuno-blotting (Haake *et al.,* 1999; Koizumi and Watanabe, 2003; AiHua *et al.,* 2011). Four conserved regions from *OmpL1* and *LipL41* have been evaluated as B cell epitopes and T cell epitopes for their potential utilization in vaccines against leptospirosis (Lin *et al.,* 2011). Also in this study immuno-blots confirmed that recombinant protein antigens reacted successfully with mice raised anti-serum indicating that recombinant *LipL41*, *Loa22* and *LipL21* may be used either alone or in combination to develop sero diagnostic ELISA or to develop a potential vaccine against leptospirosis.

Conclusion

loa22 and *lipL21* gene sequences of L. interrogans serovars Grippotyphosa and Canicola respectively were conserved in nature but *lipL41* gene sequence from *L. interrogans* serovar Grippotyphosa showed variation in nucleotide sequence which contributed to serovar evolution within the species. Antiserum raised in mice against recombinant immuno-dominant proteins LipL41, Loa22 and LipL21 successfully reacted with proteins in immuno-blot tests revealed that recombinant proteins mimic the role of natural surface exposed antigens of Leptospira. These recombinant proteins can be used for various downstream applications like as vaccine candidates and for development of ELISA for sero-diagnosis of leptospirosis.

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