

Research Article

Evaluation of Recombinant Proteins Based Indirect ELISA and Microscopic Agglutination Test for Seroprevalence Study of Feline Leptospirosis

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ABSTRACT:

Leptospirosis is worldwide zoonotic disease that is serious problem in south Indian states and now emerging in North India. The focus these days is to develop recombinant outer membrane protein based Enzyme-linked immunosorbent assay (ELISA) for seroprevalence of leptospirosis. The aim of this study was to evaluate indirect ELISA based on recombinant LipL41, Loa22, LipL21, LipL32 and OmpL1 proteins in comparison with Microscopic agglutination test (MAT). For this His-tagged recombinant proteins were expressed in DH5 α host cells and purified by Ni-NTA affinity chromatography. Hyper-immune sera raised against recombinant proteins in rabbits confirmed by dot blotting and appearance of dark dot confirm the successful antigen-antibody binding. Leptospirosis suspected 7 tiger and 3 lion sera samples screened with rLipL41+Loa22+LipL21 and rLipL41+LipL32+OmpL1 antigen combinations based indirect ELISA. Only 2 tiger samples were positive by MAT against Grippotyphosa and Icterohemorrhagae serovars in comparison to 6 sera samples positive by rLipL41 +Loa22 + LipL21 and rLipL41 +LipL32 + OmpL1 antigen combination based indirect ELISA. Out of 3 lion samples 2 were positive by MAT against Icterohemorrhagae serovar while in rLipL41 +Loa22 + LipL21 and rLipL41 +LipL32 + OmpL1 antigen combination based indirect ELISA all were positive. MAT titre values were lower than indirect ELISA titre values revealed that ELISA is more sensitive than MAT. rLipL41+LipL32+OmpL1 antigen combinations based indirect ELISA showed high titre values as compared to rLipL41+Loa22+LipL21 antigen combination based ELISA that confirmed the presence of most immunodominant protein LipL32 in the combination.

Keywords: *Leptospira*, Expression, Hyperimmune sera, Blotting, MAT, ELISA.

[I] INTRODUCTION

Leptospirosis, an anthroozoonotic infection with multisystemic involvement, is caused by the pathogenic strains of *Leptospira interrogans*. Leptospire cause life threatening zoonotic infections in almost all the domestic animals, several wild animals and humans. The most

frequent sources of infection are contaminated urine, surface water, mud and soil. Leptospirosis is a serious public health problem in both developing and developed countries [1]. Leptospirosis is considered as globally re-emerging zoonosis with marked increase in

number of cases throughout the world as reported from South East Asia (Thailand, India, Indonesia and Malaysia) and Latin America [2]. Leptospirosis is a serious problem in several south Indian states including Kerala, Karnataka, Puducherry, Tamilnadu and in Andaman & Nicobar Islands and this disease has been claimed to be an emerging zoonosis in Northern India [3] and a large number of cases have been reported from North Indian states including Delhi, Punjab and Haryana [4-6].

To prevent the spread as well as to minimize losses in terms of morbidity and mortality, it is of utmost importance to diagnose the disease quickly and to know the seroprevalence so that treatment can be started at the earliest. There are different diagnostic methods for leptospirosis that include isolation of the causative organism, visualization by dark-field microscopy, specialized staining methods, Microscopic agglutination test (MAT), Enzyme-linked immunosorbent assay (ELISA) and Polymerase Chain Reaction (PCR). Since the organism is fastidious and fragile in nature, it is difficult to isolate the organism in the laboratory [7, 8]. Dark field microscopy is carried out to visualize the organism in the clinical specimens but this also needs technical expertise.

Therefore, some sensitive and specific tests are need of the time so that the disease can be diagnosed quickly. Among these ELISA is a quantitative procedure, simple, safe and is a very suitable assay for the examination of large number of sera samples.

ELISA kits based on recombinant proteins replace the handling of highly pathogenic *Leptospira*. The most abundant proteins of *Leptospira* are LipL32, LipL36, LipL41, LipL48, LipL45, LipL21, OmpL1 and Loa22 that are pathogenic in nature [9, 10]. In the present study LipL41, Loa22, LipL21, LipL32 and OmpL1 recombinant proteins based indirect ELISA was developed and compared with gold standard Test MAT.

[II] MATERIALS AND METHODS

2.1. Cell Lysate Preparation

Leptospira interrogans serovar Canicola was procured from Regional Medical Research Centre, Indian Council of Medical Research, Port Blair, Andaman & Nicobar Islands (India). *Leptospira* culture was maintained on Leptospira Medium (LM) supplemented with Leptospira Enrichment (Himedia, Mumbai) at 29°C in BOD incubator. 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.

2.2. Expression of Recombinant Proteins

For amplification of truncated *lipL41*, *loa22*, *lipL21*, *lipL32* and *ompL1* genes, primers were designed after multiple sequence alignment of the available sequences on NCBI database (Table 1). For directional cloning of the truncated genes restriction endonuclease (RE) sites were included in the forward and reverse primers respectively (Table 1). Putative signal sequences were removed at the 5' end of the coding sequences (not included in the forward primer) of the genes. PCR reaction was set up in 25µl mixture containing 1X PCR buffer, 2.5mM MgCl₂, 200µM dNTP mix, 20pM of each of forward and reverse primers, 2.5U of Taq DNA polymerase (Fermentas Inc., Maryland USA) and ~50ng of template DNA. The PCR assay was standardized for selected five genes at the annealing temperatures as given in Table (1) to get a single band. 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*, *lipL21*, *lipL32* and *ompL1* genes were bulk amplified and gel extracted using Qiagen gel extraction kit (Qiagen, USA). pProExHT (b) prokaryotic expression vector (Invitrogen, USA) was used to ligate amplicons of *lipL32*, *lipL41*, *loa22*, *ompL1* 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 their respective restriction enzymes (Fermentas Inc., Maryland USA) and ligated at 14°C for overnight. Ligated products were transformed in DH5 α (*E.coli*) competent cells by CaCl₂ 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 their respective 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).

<insert table 1 here>

2.3. Induction of Expression

One positive clone for each gene was grown in LB broth containing ampicillin (100 μ g/ml) to the level of 0.6 OD then induced by adding 0.6 mM isopropyl β -D thiogalactopyronoside (IPTG). 2.0 ml sample was collected at 0hr then after 6hr 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.

2.4. Induction of Hyper-immune sera

Soviet-chinchilla rabbits (2 rabbits for each protein) were injected subcutaneously with recombinant proteins at 100 μ g quantity along with Freund's complete adjuvant (Santa Cruz

biotechnology, inc.), for total five doses upto 28 days at 7 day interval. 2 negative control rabbits were also included in this study. For positive control polyclonal sera were raised against heat killed *Leptospira* in 2 rabbits. For this bacterial pellet was suspended in PBS and heat killed at 121°C for 45 min using autoclave then 250 μ l of crude protein was mixed with 250 μ l of Freund's complete adjuvant and injected subcutaneous for total five doses upto 28 days at 7 day interval. At 30th day blood was collected from rabbits by heart puncture and sera were collected from clotted blood. The presence of antibodies in the sera confirmed by dot blotting and sera were further used to standardize the indirect ELISA. For dot blotting rabbit sera were used as primary antibody (1:200), goat anti-rabbit IgG HRP (horseradish peroxidase) conjugate (Santa Cruz biotechnology, inc.), as secondary antibody (1:2000) and DAB (Diaminobenzidine) as staining solution.

2.5. Collection of the clinical serum samples

Veterinary specialist from ChattBir Zoo (formally Mahendra Chaudhary Zoological Park) near Chandigarh and from Tiger Safari Zoo, Dist. Ludhiana send blood samples to diagnostic laboratory of veterinary clinic, GADVASU, Ludhiana for assessment. Out of them total 7tiger and 1lion samples, suspected for leptospirosis were collected from diagnostic laboratory for this study.

2.6. Microscopic agglutination test (MAT)

To evaluate the ELISA with gold standard test MAT, sera samples collected from tiger and lion were sent to Bacteriology & Mycology Division, IVRI, Izatnagar, Bareilly, UP (India) for MAT diagnostic test analysis.

2.7. Indirect ELISA based on recombinant proteins

Optimum antigen dilutions of LipL32, LipL41, Loa22, LipL21 and OmpL1 recombinant proteins were analyzed by checkerboard method using serial double dilutions of recombinant proteins in horizontal and hyperimmune sera dilutions in

vertical directions of the 96 well flat bottom ELISA plate (Nunc, USA). Optimum antigen concentration was selected by taking the OD values double than OD value of negative sample that lie in lower serum dilution. After the standardization of ELISA tiger and lion serum samples were screened using rLipL41+Loa22+LipL21 antigen and rLipL41+LipL32+OmpL1 antigen combinations. For this 50µl of the selected antigen concentrations were coated into plate using carbonate-bicarbonate buffer and placed at 4°C for overnight. Next day plate was washed three times with PBS-T buffer (phosphate buffer saline tween-20) and 50µl of blocking buffer (2% gelatin and 3% skimmed milk) was dispensed into wells then placed at 37°C for 2hr. After incubation plate was washed. Then wells were incubated with 50µl of feline sera that was serially double diluted 1:4 to 1:8192 using blocking solution in horizontal direction and kept at 37°C for 2hr. After that the plate was washed and goat anti-cat IgG HRP conjugate (Santa Cruz biotechnology, inc.) was put in 1:2000 dilutions into ELISA plate wells and incubated at 37°C for 2hr. In the plate positive control, negative control, conjugate control and antigen blank were also included. Again plate was washed and stained with OPD (o-Phenylenediamine) and after appearance of the yellow brown color the reaction was stopped by 3M H₂SO₄. Plate was read spectrophotometrically at 490nm on ELISA reader (BioTek). Titre was analyzed from OD reading using the standard formula- Cut off value: Mean of negative control + 3 Standard Deviation. The OD reading that was equal or above the cut off value considered as cut off titre value. The OD values above the cut off titre value were considered positive and below ones as negative. Then log₁₀ titre values were determined and compared with log₁₀ titre values of MAT.

[III] RESULT AND DISCUSSION

3.1. Expression of recombinant proteins

The growth of *Leptospira interrogans* serovar Canicola appeared within 6-7 days in the form of

turbidity. Genomic DNA was extracted using hot cold lysis method. PCR amplicons of 1028 bp, 548 bp, 472bp, 732bp and 903bp size were obtained for *lipL41*, *loa22*, *lipL21*, *lipL32* and *OmpL1* 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 restriction enzymes resulted in release of specific size inserts for the selected five genes.

SDS-PAGE analysis of induced clones resulted in a thick band of proteins as compared to un-induced controls. Purification of the proteins by Ni-NTA affinity chromatography resulted in specific band of size ~45kDa for LipL41 protein (Figure 1a), ~28 kDa for Loa22 protein (Figure 1b), ~17 kDa for LipL21 protein (Figure 1c), ~35kDa for LipL32 (Figure 1d) and ~35kDa for OmpL1 protein (Figure 1e).

<insert figure 1 here>

3.2. Confirmation of rabbit sera

For immunological characterization sera raised against recombinant proteins were separated from clotted blood. Presence of antibodies in the sera confirmed by dot blotting that resulted in dark colored dots for LipL41 (Figure 2a), Loa22 (Figure 2b), LipL21 (Figure 2c), LipL32 (Figure 2d) and OmpL1 (Figure 2e).

The serum raised in the rabbits was used to standardize the indirect ELISA.

<insert figure 2 here>

3.3. MAT titre values

Out of 7 tiger samples only 2 were positive by MAT against *Grippytyphosa* and *Icterohemorrhagae* serovars showing 1:100 titre values.

Out of 3 lion samples 2 were positive by MAT against *Icterohemorrhagae* serovar showing 1:200 and 1:400 titre values. The log₁₀ values of the titres for MAT positive samples were calculated and compared with log₁₀ values of the ELISA titre values (Table 2 and 3).

3.4. Indirect ELISA titre values

For standardization of ELISA optimum antigen dilutions for LipL41 (1:640), for Loa22 and LipL21 (1:80), for LipL32 and for OmpL1 (1:320) proteins were selected by checkerboard method. Titre values for tiger and lion sera samples with rLipL41+Loa22+LipL21 antigen and rLipL41+LipL32+OmpL1 antigen combinations based ELISA were determined and converted into log₁₀ values. After that MAT log₁₀ values compared with ELISA log₁₀ values considering only MAT positive samples (Table 2 and 3).

<insert table 2 and 3 here>

[IV] DISCUSSION

Leptospiral *lipL41*, *loa22*, *lipL21*, *lipL32* and *OmpL1* genes were amplified in truncated form to express proteins that were purified as his-tagged recombinant proteins. Sometimes his-tag lead to increase in molecular weight of proteins. Dot blotting study showed that antisera raised in rabbits successfully reacted against rLipL41, rLoa22, rLipL21, rLipL32 and rOmpL1 proteins showing that they mimic the role of natural surface exposed antigens of *Leptospira*. Similarly His6-tagged LipL21, LipL32 and OmpL1 proteins and GST tagged recombinant Loa22 protein were expressed and used to investigate the immune response against them [11, 12]. Indirect ELISA based on rLipL41+Loa22+LipL21 antigen and rLipL41+LipL32+OmpL1 antigen combinations revealed that out of the 7 leptospirosis suspected tiger samples 6 were positive. ELISA results were comparable to MAT that showed only 2 samples positive. Similarly for 3 lion samples all were positive in ELISA but only two were positive in MAT. ELISA titre values were higher than MAT titre values revealing that ELISA is more sensitive than MAT and can detect low level of antibodies present in the serum. Among the two antigen combinations rLipL41+LipL32+OmpL1 antigen combination was showing the high titre values and confirmed

the presence of most immunodominant protein LipL32 in the combination. Similarly recombinant LipL32, OmpL1 and LipL41 antigens based ELISA were developed and evaluated with MAT for diagnosis of leptospirosis and ELISA showed the high sensitivity and specificity than MAT [13, 14]. The present study revealed that ELISA make a good choice for diagnosis of leptospirosis and in future these recombinant proteins can be used as vaccine candidate.

[V] CONCLUSION

Antiserum raised in rabbits against recombinant immunodominant LipL41, Loa22, LipL21, LipL32 and OmpL1 proteins successfully reacted with proteins in dot blot tests and mimic the role of natural surface exposed antigens of *Leptospira*. In comparison to MAT indirect ELISA showed high titre values and high sensitivity. High titre value of rLipL41+LipL32+OmpL1 antigen based ELISA confirm the presence of most immunodominant protein LipL32 in the combination.

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Truncated Gene	Primer sequence (5'- 3')	RE sites	Amplicon in bp	Annealing Temp.
<i>lipL41</i>	F: <u>cgcccatg</u> gccgcagctaca R: cg <u>ctcgag</u> ttactttgegt	<i>NcoI</i> <i>XhoI</i>	1028	60°C
<i>loa22</i>	F: <u>cgcccatg</u> gctgctcctctg R: <u>cgctcgag</u> ttattgttggtg	<i>NcoI</i> <i>XhoI</i>	548	55°C
<i>lipL21</i>	F: <u>aaccatgg</u> acacaggacaaaaagacg R: <u>aactcgag</u> acgagagcctctacca	<i>NcoI</i> <i>XhoI</i>	472	60°C
<i>lipL32</i>	F: <u>cgcccatg</u> gcc aaaagctctttgtctgagcgag R: <u>cgctcgag</u> ttacttagtcgctcagaagcagc	<i>NcoI</i> <i>XhoI</i>	732	55°C
<i>OmpL1</i>	F: <u>cccgatcc</u> atgatccgtaacataagtaagg R: <u>cccgatcc</u> cagcctaagtgcaaaaacatagc	<i>BamHI</i> <i>HindIII</i>	903	50°C

Table 1: Primers designed for amplifying *lipL41*, *loa22*, *lipL21*, *lipL32* and *OmpL1* genes by polymerase chain reaction (PCR). Underlined were restriction enzyme (RE) sites

Tiger/Lion sample No.	MAT	MAT titre	ELISA Titre
1 (Tiger)	-	-	3.010299957
2 (Tiger)	-	-	2.709269961
3 (Tiger)	1:100 Grippo	2	2.709269961
5 (Tiger)	-	-	1.505149978
6 (Tiger)	-	-	3.311329952
7 (Tiger)	-	-	2.408239965
8 (Lion)	-	-	3.311329952
9 (Lion)	1:200 Ictero	2.301029996	3.311329952
10 (Lion)	1:400 Ictero	2.602059991	3.010299957
11 (Tiger)	1:100 Ictero	2	2.709269961

(-)Negative

Table 2: Comparison of MAT and LipL41 +Loa22 + LipL21 antigen combination based ELISA log10 titres for feline samples

Tiger/Lion sample No.	MAT	MAT titre	ELISA titre
1 (Tiger)	-	-	3.311329952
2 (Tiger)	-	-	3.311329952
3 (Tiger)	1:100 Grippo	2	3.311329952
5 (Tiger)	-	-	1.806179974
6 (Tiger)	-	-	3.311329952
7 (Tiger)	-	-	3.311329952
8 (Lion)	-	-	3.311329952
9 (Lion)	1:200 Ictero	2.301029996	2.408239965
10 (Lion)	1:400 Ictero	2.602059991	3.612359948
11 (Tiger)	1:100 Ictero	2	3.612359948

(-)Negative

Table 3: Comparison of MAT and LipL41 +LipL32 + OmpL1 antigen combination based ELISA log10 titre values for feline samples

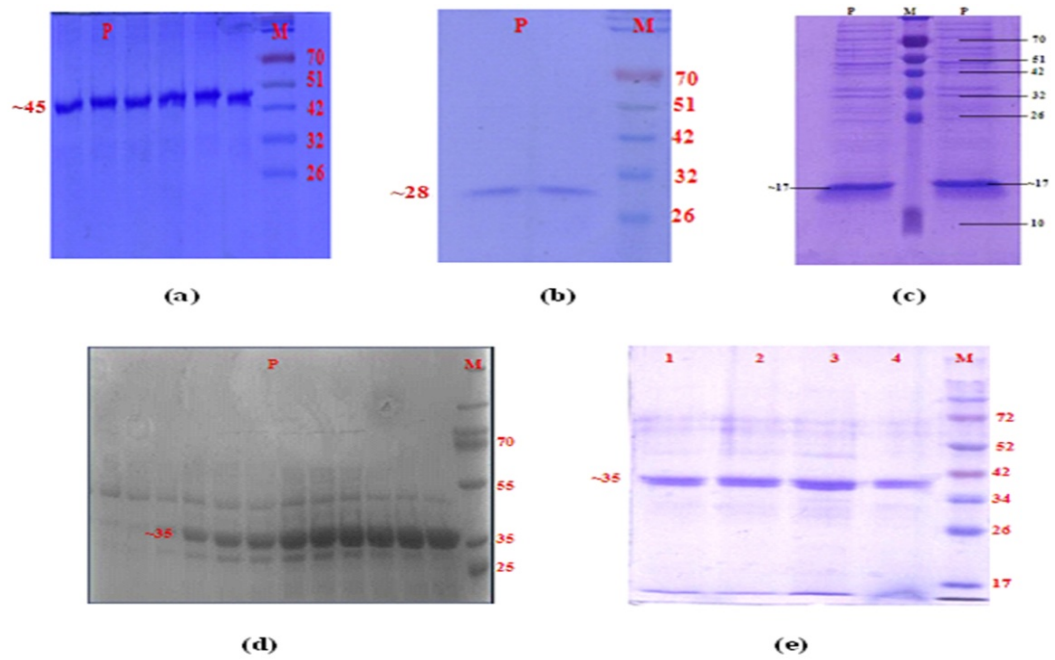


Figure 1: Purified recombinant proteins by Ni-NTA affinity chromatography. (a) LipI41 protein; (b) Loa22 protein; (c) LipL21 protein; (d) LipL32 protein; (e) OmpL1 protein. Lane (P) Protein in kDa; Lane (M) Multicolour broad range protein ladder

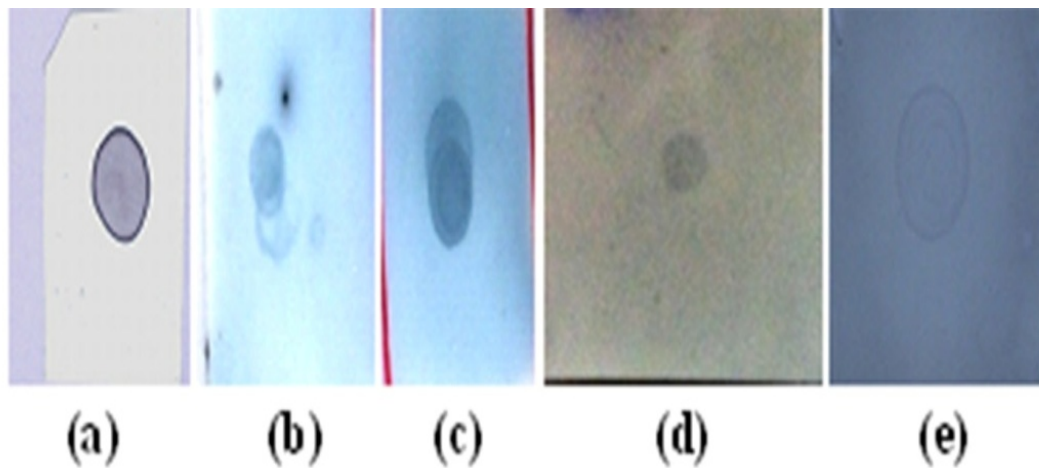


Figure 2: Dot blot of recombinant proteins with rabbit hyperimmune serum. (a) LipI41 protein; (b) Loa22 protein; (c) LipL21 protein; (d) LipL32 protein; (e) OmpL1 protein.