DEVELOPMENT OF RECOMBINANT LEPTOSPIRAL ANTIGEN FOR THE DIAGNOSIS OF LEPTOSPIROSIS

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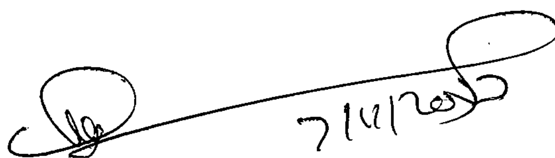
CERTIFICATE

This is to certify that the thesis entitled **"DEVELOPMENT OF RECOMBINANT LEPTOSPIRAL ANTIGEN FOR THE DIAGNOSIS OF LEPTOSPIROSIS"** submitted in partial fulfillment for the requirements of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOTECHNOLOGY** to the Tamil Nadu Veterinary and Animal Sciences University, Chennai, is a record of bonafide research work carried out by **SOHINI DEY** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.



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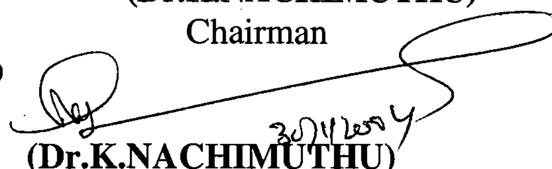

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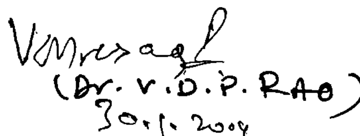

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Abstract

ABSTRACT

Title	:	**DEVELOPMENT OF RECOMBINANT LEPTOSPIRAL ANTIGEN FOR THE DIAGNOSIS OF LEPTOSPIROSIS**
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Leptospirosis is a zoonosis of worldwide significance, caused by infection with pathogenic *Leptospira* species. There is an urgent need for development of new diagnostic strategies for leptospirosis. The standard serological test, the microscopic agglutination test (MAT), is restricted to specialized and well-equipped laboratories. Moreover, the MAT requires detailed knowledge of the locally occurring strains, as the predominant serovars have to be selected for use as antigen. Hence, the recombinant Lip L32 was developed as antigen, the sequence and expression of which is highly conserved among pathogenic *Leptospira* species.

Primers were designed with built-in restriction enzyme sites for amplification of a portion of the major outer membrane protein, the Lip L32. The amplicon of *Leptospira pomona* strain Pomona was cloned into pBluescript

II KS (+) vector and expressed from pPro EX HT 'a' vector following transformation into DH5α cells. The expressed protein reacted with antiserum raised against *pomona* but could not react with antiserum raised against *patoc* as analysed by Western Blot technique. The recombinant fusion protein tagged with polyhistidine was purified by affinity chromatography. The concentration of the purified antigen was determined and the recombinant Lip L32 protein was utilized in different assays for the diagnosis of Leptospirosis.

An attempt was made to detect the antigen in acute infection from clinical samples using the recombinant Lip L32 antigen by Indirect competitive assay. But satisfactory results could not be obtained due to low concentration of antigen in the samples.

Recombinant antigen-based tests like single serum dilution enzyme linked immunosorbent assay (ELISA), an easy-to-perform Dipstick ELISA and a rapid and simple Latex agglutination test kits were developed for the first time to facilitate the use of the recombinant antigen in canine and human serodiagnosis in the developing countries.

Keywords: *Leptospira* - recombinant Lip L32 - cloning - expression - diagnosis - leptospirosis.

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LIST OF ABBREVIATIONS

OMP	-	Outer membrane protein
rLip L32	-	Recombinant Lip L32
LPS	-	Lipopolysaccharide
DFM	-	Dark field microscopy
PFGE	-	Pulsed field gel electrophoresis
RIA	-	Radio immunoassay
MAT	-	Microscopic agglutination test
ELISA	-	Enzyme-linked immunosorbent assay
LAT	-	Latex agglutination test
PCR	-	Polymerase chain reaction
EMJH	-	Ellinghausen McCullough Johnson and Harris medium
DNA	-	Deoxyribouncleic acid
MEGA	-	Molecular Evolutionary Genetic Analysis
PNT	-	Positive - negative threshold
HRP	-	Horse Radish Peroxidase
Ig	-	Immunoglobulin
bp	-	base pair
kDa	-	Kilo Dalton

Blocking buffer

Tris - HCl (pH 8.0)	10 mM
NaCl	150 mM
Tween 20 v/v	0.05%
BSA	3%

Blocking buffer was stored at 4°C and can be used several times when sodium azide was added to a final concentration of 0.05% (w/v) to inhibit the growth of microorganisms.

3.1.15 Reagents for Enzyme linked immunosorbent assay (ELISA)

Recombinant Lip L32 antigen

Rabbit anti-human and anti-dog IgG - HRP conjugate (Bangalore Genei)

Human and dog serum samples

Carbonate-bicarbonate buffer (pH 9.6)

Sodium Carbonate	1.59 g
Sodium bicarbonate	2.93 g

Distilled water was added, pH was adjusted to 9.6 and volume was made upto 1 litre.

Sodium citrate buffer (pH 4.2)

Sodium citrate 14.705 g

Distilled water was added, pH was adjusted to 4.2 and volume was made upto 1 litre.

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Introduction

1. INTRODUCTION

Leptospirosis, a zoonosis with a worldwide distribution, is an acute febrile illness caused by spirochaetes of the pathogenic *Leptospira interrogans*. Leptospirosis affects a wide range of hosts including human, domestic and wild animal species (Faine, 1994; Hartskeerl and Terpstra, 1996). Human are accidental hosts and become infected through contact with an environment contaminated by the urine of a shedder host, such as rodents.

Infections are typically transmitted to human through contact with water or soil contaminated with the urine of infected animals. Warm and wet climate with salinity of soil favours the growth of *Leptospira*. The occurrence of large outbreaks of leptospirosis is usually observed following severe floods. The incidence is significantly higher in tropical countries than in temperate regions. This is mainly due to longer survival of leptospires in the warm, humid conditions. Recently, in India, an outbreak has been recorded during the post-cyclone investigation in Orissa (WHO, 2000).

Clinical recognition of leptospirosis is difficult because leptospires can affect many different organ systems, resulting in a wide variety of clinical presentations. Consequently, leptospirosis is often misdiagnosed as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis, or gastroenteritis. In domestic animals it is a disease of major economic importance as it affects productivity and results in weakness, abortion or even death. Timely diagnosis of leptospirosis is essential because prompt, specific treatment, as early in the illness as possible is important to ensure a favourable clinical outcome.

Laboratory confirmation is of utmost importance and until now serology has been the cornerstone of different laboratory diagnosis. The standard serologic test, microscopic agglutination test (MAT), uses live strains for the serodiagnosis of leptospirosis and the test has a high sensitivity and can be used for classification (Faine, 1982). Unfortunately, the application of the MAT is laborious and requires expertise. Moreover, application of the test also requires detailed knowledge of the locally occurring strains, as the predominant serovars have to be selected for use as antigens. The use of MAT is inadequate for rapid case identification since it can only be performed in a few reference laboratories and requires analysis of paired sera to achieve sufficient sensitivity. Dependence upon the MAT results in delays in establishing the cause of outbreaks. Enzyme-linked immunosorbent assay (ELISA) and other rapid serologic tests like Dipstick ELISA, Latex Agglutination Tests based on whole cell leptospiral antigen preparations have been developed to screen for leptospiral infection. Recombinant protein - based serologic tests may achieve high sensitivity and specificity because of the high concentration of immuno reactive antigens which can be used in assays and the lack of non-specific moieties present in whole-cell preparations. Lip L32, a major leptospiral outer membrane protein (OMP) has been identified as a serodiagnostic marker for screening leptospiral infection (Haake *et al.*, 2000). It is possible to express and purify Lip L32 as a recombinant fusion protein in a form suitable for diagnostic format. The protein is expressed only in the pathogenic leptospiral species and conserved among more than 200 serovars of *Leptospira.* The present study was undertaken to develop a recombinant Lip L32 protein from *Leptospira* as antigen for rapid diagnosis of Leptospirosis in human and dogs with the following objectives:

1. To identify and purify the gene encoding the leptospiral outer membrane protein (OMP).

2. To clone and express the OMP gene into a suitable cloning and expression vector system.

3. To isolate and purify the expressed recombinant leptospiral OMP antigen.

4. Use of purified leptospiral recombinant antigen in test systems like ELISA, Dipstick ELISA and Latex agglutination test for detection of leptospiral antibodies in serum samples.

5. To investigate the possibility of using leptospiral recombinant antigen for leptospiral antigen detection in clinical samples by Indirect competitive assay.

Review of Literature

2. REVIEW OF LITERATURE

Spirochaetes of the genus *Leptospira* are the causative agents of Leptospirosis, an emerging zoonosis encountered worldwide. Although leptospirosis has a world wide distribution, it is most common in tropical and rural areas (Farr, 1995; Ko *et al.*, 1999). Leptospires persist in the proximal renal tubules of kidneys in reservoir hosts and are shed in the urine into the surrounding environment. Many animals serve as reservoir hosts and transmit the pathogen directly or indirectly to human. The disease is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality. It is likely that the wide distribution of *Leptospira* species reflects the ability to survive in diverse environmental conditions combined with genetic adaptation, which is reflected in the plasticity of the leptospiral genome (Zuerner *et al.*, 1993).

2.1 Classification

2.1.1 Serological classification

Prior to 1989, the genus *Leptospira* was divided into two species, *Leptospira interrogans*, comprising all pathogenic strains and *Leptospira biflexa,* containing the saprophytic strains isolated from the environment. Both are again divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen (Johnson and Faine, 1984). At least 268 serovars and 17 species of *Leptospira* and *Leptonema* have been described to date (Matsunaga *et al.*, 2002). The antigenic basis for serovar specificity resides in the carbohydrate side chain structure of lipopolysaccharide (Bulach *et al.*, 2000).

2.1.2 Genotypic classification

DNA hybridization studies led to the definition of 16 genomospecies (Yasuda *et al.,* 1987). An additional species, *Leptospira fainei,* has been described which contained a new serovar, *hurstbridge* (Perolat *et al.,* 1998). This genetic classification, which has been based on DNA homology and did not follow distinction or clustering based on antigenic similarities, divided leptospiral strains into four non-pathogenic species *Leptospira biflexa, Leptospira meyeri, Leptospira parva* and *Leptospira wolbachii* and seven pathogenic species *Leptospira interrogans, Leptospira borgpetersenii, Leptospira weilii, Leptospira noguchii, Leptospira santarosai, Leptospira inadai* and *Leptospira kirschneri* (Yasuda *et al.,* 1987; Ramadass *et al.,* 1992).

2.2 Prevalence Of Leptospirosis

Investigation in various parts of the world have revealed that leptospirosis is widely distributed mainly in marshy areas with a wide variety of rodent carriers. The dispersion of leptospires is related to specific environmental conditions particularly those, which bring infected animal, water and mud together (WHO, 1967). A number of well-organized environmental factors such as moisture, warmth, soil and water pH around neutrality, have favoured the survival of leptospires (Galton *et al.,* 1958; Sullivan, 1974).

There is an increased incidence of canine leptospirosis during late summer and early fall and in the southern semitropical belt of the United States (Ward, 2002). Disease outbreaks often increase after periods of flooding or increased rainfall.

The incidence and prevalence of serovars differ considerably among the countries and even within the country (Michna, 1970). This often accounts for the incidence of a particular serovar in the wild rodents and livestock in the same area. Each serovar has a preferential host, but cross infection are not uncommon.

2.2.1 Prevalence in India

In India, leptospirosis is known to occur for a long time. Ever since the beginning of this century the literature emanated from India proved that this disease is persisting continuously in main land India and Andaman Islands.

Taylor and Goyal (1931) were probably the first to report the prevalence of jaundice among human beings and they isolated strains of *andamana and grippotyphosa* in Andaman Islands.

Ayyar (1932) first reported the incidence of canine leptospirosis in India, while studying an outbreak of jaundice among dogs in Madras city. Four dogs were reported to be seropositive for *Leptospira interrogans* serovar *icterohaemorrhagiae*. Adinarayanan *et al.* (1960) reported an outbreak of leptospirosis in buffaloes and Sindhi heifers in Uttar Pradesh. Sawhney and Saxena (1967) observed the prevalence of leptospiral infection in cattle and buffaloes in Madhya Pradesh. In this study, 12.2% of cattle and 21.86% of buffaloes were found positive by microscopic agglutination test.

Rajasekhar (1968) reported that sixty seven out of one hundred and fifty cattle sera examined had leptospiral agglutinins, when subjected to macroscopic slide agglutination test.

Krishnappa *et al.* (1980) collected a total of 337 serum samples from people living around Bangalore city and subjected them to an agglutination lysis test using live leptospiral cultures as antigens and their report showed the highest incidence amongst agricultural workers, dairy men and veterinarians.

Ratnam *et al.* (1983) observed that 16 out of 40 buffaloes were positive for *Leptospira autumnalis* in an outbreak in a village near Chennai. Singh and Uppal (1983) found leptospiral antibodies in 42 of 739 cattle samples in Andhra Pradesh, 4 of 18 in Assam, 9 of 70 in Gujarat, 1 of 20 in Himachal Pradesh, 22 of 464 in Maharashtra, 1 of 13 in Orissa, 11 of 175 in West Bengal, 9 of 32 in Kerala and 9 of 92 in Tamil Nadu. The leptospiral antibodies observed most often were against *Leptospira interrogans* serovars *hebdomadis* and *autumnalis*.

Venkataraman and Nedunchelliyan (1992) reported that 50.5% of human sera, 21.3% of canine sera, 25% of rat sera and 41.6% of bandicoot sera were found positive for leptospiral antibodies in an outbreak that occurred in Chennai city.

Ratnam *et al.* (1994) have done a pilot study on the prevalence of leptospirosis in Tamil Nadu State. They reported that *pomona* and *autumnalis* are prevalent serovars amongst human population in the Southern and Northern districts of Tamil Nadu, respectively.

Natarajaseenivasan and Ratnam (2000) isolated 12 leptospiral isolates from dead albino mice, dead wistar rats, field and house rats, sheep and ailing human urine samples and typed them as serovar *javanica* of the serogroup Javanica and 2 human isolates as serovar *autumnalis* and *canicola*.

2.2.2 Global scenario

Leptospirosis is presumed to be the most widespread zoonotic disease in the world. It is a sporadic disease in people in the United States, with outbreaks occurring in occupational or recreational settings. Veterinarians are considered at risk, along with farmers, workers in slaughter facilities, animal researchers and sewer system workers (Campagnolo *et al.,* 2000). A recent outbreak in Baltimore was associated with potential exposure to rat urine in inner city alleys. 19 of 21 rats trapped in the region were PCR positive for leptospiral organisms (Vinetz *et al.,* 1996).

Harkin *et al.* (2003) compared PCR testing of urine samples, serologic testing, and bacterial culture of urine to determine prevalence of urinary shedding of leptospires in dogs. They concluded that irrespective of health status, 8.2% of dogs were shedding pathogenic leptospires. Serologic testing was a poor predictor of urinary shedding. Clinically normal dogs that shed leptospires may pose a zoonotic risk to their owners.

2.3 Biology Of Leptospires

Leptospires are thin, flexible, filamentous (0.1 to 0.2 µm wide x 6 to 12 µm long) bacteria(Langston and Heuter, 2003) but occasional cultures may contain much longer cells (Levett, 2001). The cells have pointed ends, either or both of which are usually bent into a distinctive hook. Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space (Swain, 1957). Haake *et al.* (2000) reported that leptospires have a typical double membrane structure in common with other spirochaetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane (OM). Three classes of leptospiral OM proteins (OMPs) had been described *viz.,* transmembrane, lipoprotein and peripheral membrane proteins. The lipoproteins are anchored to the OM by fatty acids attached to an amino-terminal cysteine (Haake *et al.,* 2000). Analysis of the detergent phase of Triton X-114 extracts of *Leptospira kirschneri* intrinsically labelled with tritiated palmitate indicated that the leptospiral OM contains at least five lipoproteins (Shang *et al.,* 1996). The genes encoding three leptospiral OM lipoproteins, Lip L32, Lip L36 and Lip L41 had been described (Haake *et al.,* 1998). Lip L32 is known to be the most prominent protein in the leptospiral protein profile and is an immunodominant antigen during human leptospirosis (Haake *et al.,* 2000). This 32 kDa antigen was identified as the major leptospiral outer membrane protein whose expression is restricted to pathogenic *Leptospira* species (Haake *et al.,* 2000).

2.4 Diagnostic Methods

Demonstration of leptospires, in clinical samples like urine and blood or leptospiral antibodies in serum is used for confirmation of the diseases.

2.4.1 Microscopic demonstration

Leptospires may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (Turner, 1970). A positive result is dependent on the observation of intact leptospires, which are often confused with proteinaceous filaments known as pseudo-leptospires (Rahman and Mairs, 1979; Waitkins and Zochwski, 1990). Microscopy of blood is of value only during the first few days of the acute illness.

Staining methods have been applied to increase the sensitivity of direct microscopic examination. These have included immunofluorescence staining of bovine urine (Hodges and Ekdahl, 1973), water and soil (Henry *et al.*, 1971) and immunoperoxidase staining of blood and urine (Terpstra *et al.*, 1985). A variety of histopathological strains have been applied to the detection of leptospires in tissues. Leptospires were first visualized by silver staining (Stimson, 1907). Gangadhar and Rajasekhar (1998) developed a modified silver impregnation staining for leptospires. Immunofluorescence microscopy is used extensively to demonstrate leptospires in veterinary specimens (Ellis *et al.*, 1982).

2.4.2 Antigen detection

Detection of leptospiral antigens in clinical materials would offer greater specificity than dark-field microscopy while having the potential for greater sensitivity. Radio immunoassay (RIA) could detect 10^4 to 10^5 leptospires/ml and an enzyme linked immunosorbent assay (ELISA) could detect 10^5 leptospires/ml, but counter current immunoelectrophoresis and staphylococcal coagulation were much less sensitive (Adler *et al.,* 1982). A double sandwich ELISA could detect 10^4 leptospires of serovar *hardjo* but was less sensitive for other serovars (Champagne *et al.,* 1991). Immunomagnetic antigen capture was combined with fluoroimmunoassay to detect as few as 10^2 leptospires/ml in urine of cattle infected with leptospiral serovars (Yan *et al.,* 1998).

2.4.3 Serological diagnosis

Antibodies were detectable in the blood approximately 5 to 7 days after the onset of symptoms (Levett, 2001).

2.4.3.1 Macroscopic agglutination test

In this test, a concentrated suspension of killed leptospires would agglutinate in the presence of agglutinins into clumps that are visible to the naked eye. Stoenner (1953) developed a macroscopic capillary tube agglutination test using antigens prepared by suspending formalin-killed leptospires in buffered hypertonic sodium chloride solution. Later, Galton *et al.* (1958a) developed a macroscopic slide test with different antigen pools, however the test was considered less sensitive than Microscopic Agglutination Test (MAT).

2.4.3.2 Microscopic agglutination test

The reference method for serological diagnosis of leptospirosis is the MAT, in which patient sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination, and the titres determined. Formerly the method was known as the agglutination lysis test because of the formation of lysis balls or lysis globules (Van Thiel, 1948) of cellular debris in the presence of high-titred antiserum. However, these are tightly agglutinated clumps of leptospires containing live cells and not debris (Turner, 1968).

The MAT is a complex test to control, perform and interpret (Turner, 1968). Live cultures of all serovars required for use as antigens must be maintained. The repeated weekly subculture of large numbers of strains present hazards for laboratory workers and laboratory acquired infections have been reported (Alexander *et al.,* 1952; Pike, 1976). Stoenner (1972) reported that the MAT was the test of choice when used as a screening test in investigating the possibility of *Leptospira hardjo* infections in herds of cattle. Blackmore (1985) reported that the sensitivity declined when animals were tested a considerable time after infection. Effler *et al.* (2002) reported that because a four fold rise in titre between acute and convalescent phase samples is necessary for serologic confirmation, the MAT is not useful for guiding clinical management early in the course of illness.

The MAT is read by dark field microscopy. The end point is the highest dilution of serum at which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospires compared to the control suspension (Faine, 1982).

Formalized antigens have been used in the MAT to overcome some of the difficulties associated with the use of live antigens. Titres obtained with these antigens are somewhat lower, and more cross reactions are detected (Palmer *et al.,* 1987).

Faine (1982) reported that the MAT is also the most appropriate test to employ in epidemiological serosurveys, since it can be applied to sera from any animal species and the range of antigens used can be expanded or decreased as required. It is usual to use a titre of ≥ 100 as evidence of post exposure. A single titre of greater than 1:800 in an unvaccinated dog with classic signs of Leptospirosis provides presumptive evidence of leptospirosis, although some would suggest using a cut-off of 1:3200 or higher (Bolin, 1996). The MAT data could give a general impression about which serogroups are present within a population (Levett, 2001).

Natarajaseenivasan *et al.* (2002) isolated *Leptospira* from rice mill workers in Salem, South India. The most predominant serogroup identified by MAT was Autumnalis in rice mill workers and all animal populations. The other serogroups that reacted in MAT were Icterohaemorrhagiae, Australis,

Grippotyphosa and Patoc. The rice mills of Salem, which had large rodent populations, various animals lived in close proximity, wet environment and unprotected exposure of the workers to the environment, constituted an ideal setting for transmission of leptospirosis and could be an epidemiological niche of leptospirosis.

Levett (2003) identified the usefulness of MAT as a predictor of the infecting serovar in patients with serum for leptospirosis. He concluded that serologic analysis appeared to be of little value for the identification of the infecting serovar in individual cases of leptospirosis in humans. Presumptive serogroup reactivity data should be used only to gain a broad idea of the serogroup present at the population level.

2.4.3.3 Enzyme linked immunosorbent assay (ELISA)

Adler *et al.* (1980) used ELISA to detect *Leptospira*-specific IgM and IgG in the sera of patients infected with leptospiral serovars *hardjo, pomona* or *copenhageni*. All patients produced specific IgM and IgG antibodies detectable by ELISA. IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness (Cumberland *et al.*, 1999).

Adler *et al.* (1981) compared ELISA with the standard MAT as a method for detecting antibodies against *Leptospira interrogans* serovar *hardjo* in sheep. Even though the two tests measured different antibody systems, they found 95% correlation and concluded that the ELISA would be a useful test for screening large numbers of sera for antibodies to *L.interrogans* serovar *hardjo*.

Biancifiori and Cardaras (1983) performed the ELISA test for detection of antibodies to leptospirosis in domestic animals using staphylococcal protein-A coupled to peroxidase. MAT and ELISA were compared in a total of 48 positive swine sera and 100% agreement was obtained. Sixteen sera samples from clinically suspected cases of leptospirosis showed that ELISA was more sensitive and precocious than MAT in detecting specific antibodies.

Waltman and Dawe (1983) applied a genus-specific ELISA for the detection of anti-leptospiral antibodies in swine sera. Results of the ELISA directly correlated with the results of MAT. They calculated the ELISA antibody titre as 1:25 comparable with the MAT titre of 1:100, indicative of an active leptospiral infection. They also found that the ELISA had a sensitivity of 100%, specificity of 86.4% and predictive accuracy of 91.3%.

Hartman *et al.* (1984) developed and evaluated ELISA to detect antibodies to *L.interrogans* serotype *canicola* in dogs. Comparison of the ELISA with the MAT showed that, during the first two weeks after an experimental infection with serotype *canicola*, ELISA detected antibody at higher dilutions than the MAT. After the second week post-infection both tests detected antibody at almost equal titres. They found that both the outer envelope and pellet antigen of *L.biflexa* strain Patoc I could be used as cross-reacting antigen in the ELISA. They also suggested that the ELISA would be useful as a screening test.

Leptospira interrogans serovars *pomona*, *hardjo* and *tarassovi* were each used to inoculate cattle. Three-hundred and ninety-nine sera collected from the inoculated animals and from a control group over a 3-month period were

tested using the MAT and ELISA. The ELISA detected specific IgM antibody against the serovars in all infected cattle 1 week after inoculation. This IgM antibody persisted in most of the animals for 3-5 weeks. Specific IgG antibody appeared at the same time or just after IgM, but persisted for much longer (Cousins *et al.*, 1985).

Terpstra *et al.* (1985a) used the ELISA to detect specific IgM and IgG in sera from humans with current or post leptospirosis. A serological pattern of a high IgM titre (> 1280) or moderaltely increased IgM (160-640) in conjunction with a low IgG titre (< 20), with serovar *copenhageni* antigen was characteristic for approximately two-thirds of the sera from serovar *icterohaemorrhagiae* patients, in the first two months of the disease. They observed that in post infections, the IgG titres were clearly higher with the homologous antigen.

Cho *et al.* (1989) prepared the outer sheath antigen from *Leptospira interrogans* serovars *pomona, sejroe* and *hardjo*. The specificity of this assay, which were negative in the MAT using seven serovars, was 99.4%. The relative sensitivity of the test was 100%.

Bercovich *et al.* (1990) compared the ELISA with the MAT for the diagnosis of *Leptospira interrogans* serovar *hardjo* infection in cattle. They used glutaraldehyde in the ELISA to couple sonicated *hardjo* antigen to the microtiter plate. They concluded that the ELISA is an alternative to the MAT for diagnosing leptospirosis.

Gussenhoven *et al.* (1997) studied a dipstick assay for the detection of Leptospira - specific IgM antibodies in human serum samples. They observed a high degree of concordance between the results of the dipstick assay and an IgM ELISA. Application of the dipstick assay for the detection of acute leptospirosis enabled the accurate identification, early in the disease. Analysis of a second serum sample was recommended, in order to determine seroconversion or increased staining intensity. They observed some cross-reactivity for sera from patients with diseases other than leptospirosis, which should be taken into account in the interpretation of test results.

Ribotta *et al.* (2000) in their study used a genus-specific, heat stable antigenic preparation from *Leptospira interrogans* serovar *pomona* in an ELISA for the detection of leptospiral antibodies in dog sera. The ELISA showed a relative specificity of 95.6% with 158 dog sera, which were negative at a dilution of 1:200 in the MAT for serovars *pomona, bratislava, icterohaemorrhagiae, autumnalis, hardjo* and *grippotyphosa.* The relative sensitivity of this assay with 21 dog sera that revealed the MAT titres of ≥ 100 to different serovars were 100%. They concluded that this ELISA is sufficiently sensitive test to be used as an initial screening test for the detection of leptospiral antibodies in canine sera with subsequent confirmation of positive test results with the MAT.

Levett *et al.* (2001) showed a commercial IgM dot ELISA dipstick to be as sensitive as a microtitre plate IgM ELISA. This test could be used for rapid diagnosis of acute leptospiral infection.

Flannery *et al.* (2001) evaluated the diagnostic utility of five recombinant antigens (Lip L32; Omp L1; Lip L41, Hsp 58 and Lip L36) in ELISA for serodiagnosis of leptospirosis. In paired sera from 50 cases of leptospirosis confirmed by MAT, IgG but not IgM, reacted with the recombinant leptospiral proteins. The recombinant Lip L32 IgG ELISA had the highest sensitivities in the acute (56%) and convalescent (94%) phases of leptospirosis. Recombinant Lip L32 IgG ELISA demonstrated 95% specificity among 100 healthy individuals, and specificities ranging from 90 to 97% among 30 dengue cases initially thought to be leptospirosis. Among 39 venereal disease research laboratory test-positive individuals and 30 Lyme diseases patients, 13 and 23% of sera, respectively reacted positively with the recombinant Lip L32 antigen. These findings indicated that recombinant Lip L32 might be a useful antigen for the serodiagnosis of leptospirosis.

Effler *et al.* (2002) reported that the definitive serologic diagnostic assay, the MAT is time consuming and difficult to conduct. They evaluated eight rapid screening tests for acute leptospirosis in Hawaii. The median number of days between illness onset and specimen collection was 9. The overall sensitivity, for each test was as follows: indirect haemagglutination assay was 29%, INDX Leptospira Dip-S-Tick was 52%, Biognost IgM IFA test was 40%, Biolisa IgM ELISA was 48%, Leptospira IgM ELISA was 36%, SERION ELISA classic *Leptospira* was 48%, LEPTO Dipstick was 34% and Biosave latex agglutination was 86%. This was the most comprehensive field trial leptospirosis screening tests reported to date. The data indicated that IgM detection tests have limited utility for diagnosis of leptospirosis during the initial evaluation of patients seen in Hawaii, a time when important therapeutic decisions are to be made. They reported that improved leptospirosis screening tests are needed.

Abdulkadar *et al.* (2002) listed 35 hospitalized patients from the 15th day to the 12th month of symptoms for ELISA-IgM, IgG and IgA specific antibody detection. According to their 1st IgG titer, the patients were divided into: group 1 (n = 13) titre > 1:400 (positive) and group 2 (n = 22) titre ≤ 1:400 (negative). Throughout the study, IgG and IgA titre remained higher in group 1. They concluded that the severity of Weil's disease may be associated with the intensity of the humoral immune response to leptospire.

Bajani *et al.* (2003) evaluated four rapid tests namely microplate immunoglobulin M (IgM) ELISA, an indirect haemagglutination assay (IHA), an IgM dipstick assay (LDS) and an IgM dot-ELISA dipstick test (DST). They compared the performance of each with that of the current standard, MAT. A good overall correlation with the MAT was obtained for each of the assay, with the highest concordance being with the DST. The best correlation was between ELISA and DST. False positive LDS results were frequent in sera from individuals with Epstein-Barr virus, HIV and periodontal disease and from healthy workers. The ease of use and significantly high sensitivity and specificity of DST and ELISA made these good choices for diagnostic testing.

2.4.3.4 Latex agglutination test

The use of MAT had been restricted to specialized and well equipped laboratories. The Latex Agglutination Test (LAT) expected for use as a practical and rapid adjunct could be used for serodiagnosis of human and animal leptospirosis. LAT for the detection of leptospirosis had been described in the past (Muraschi, 1959).

Petchchlai *et al.* (1990) evaluated indirect haemagglutination and latex agglutination test for screening human leptospirosis. They found that LAT is one of the most rapid tests for leptospirosis.

Ramadass *et al.* (1999) standardized a rapid, semi-quantitative LAT for the detection of leptospiral antibodies in serum samples of man and animals. The efficacy of the LAT was compared with the plate ELISA. The rapidity, simplicity and economics of the LAT were found to fulfill the requirements of a screening test for leptospiral antibodies.

A newly developed latex agglutination assay for the detection of genus-specific leptospiral antibodies in human sera was evaluated (Smits *et al.*, 2000). The assay was performed by mixing, on an agglutination card, serum with equal volumes of stabilized antigen-coated, dyed test and control latex beads and was read within 2 minutes. The LAT was evaluated with groups of serum samples from patients with leptospirosis and control patients from Hawaii, the Seychelles, Thailand and the Netherlands. The mean overall sensitivity was 82.3% and the mean overall specificity was 94.6%. The assay was easy to perform and did not require special skills or equipment. This assay was suitable for use even at the peripheral level of a health care system as a rapid screening test for leptospirosis.

Verma (2001) conducted LAT using *pomona* and *hardjo* antigens. He found LAT to be rapid, simple and economically viable technique. LAT results were compared with MAT and sensitivity and specificity for human samples were found to be 84.90 and 71.42 percent, respectively. In canine samples, sensitivity and specificity of LAT were found to be 81.81 and 70.00 percent, respectively.

Vijaychari *et al.* (2002) evaluated a new card agglutination test. Lepto Dri Dot developed by the Dutch Royal Tropical Institute for the rapid diagnosis of leptospirosis. The test consisted of coloured latex particles activated with a broadly reactive leptospiral antigens, that was dried onto an agglutination card. The test was performed in the field conditions in the Andaman Islands. The test results were compared with blood culture, MAT and IgM ELISA. Both Dri Dot and ELISA showed good agreement with the standard diagnostic criteria after the first week of illness. This new test does not require special storage or sophisticated equipment and can be performed by relatively low skilled personnel.

2.4.3.5 Polymerase chain reaction

Hookey (1992) developed the polymerase chain reaction (PCR) to detect *Leptospira* infection with the primers which amplified the 5'-region of 16S rDNA. They found that isolates of leptospires from clinical sources gave a positive reaction than those from surface waters which did not show amplification.

Bal *et al.* (1994) amplified leptospires from urine samples and suggested that PCR analysis of urine could be more successful for early diagnosis of leptospirosis than PCR analysis of serum.

Zuerner *et al.* (1995) developed a PCR based assay for typing *L.interrogans sensu lato* serovars. They designed the assay to exploit the presence of the number of copies of the leptospiral insertion sequence, *IS 1533*

and *IS 1533*-like sequences in the genomes of most leptospiral serovars. Primers were designed to amplify DNA of unknown sequence between closely placed insertion sequences. DNA from different serovars showed the presence of different sized products, thus enabling the serovars to be identified. Genetic variation within the same isolates was also demonstrated.

Woo *et al.* (1997) identified pathogenic *Leptospira* genospecies by continuous monitoring of flurogenic hybridization probes. Sequence analysis enabled *Leptospira* genus specific primers and pathogenic specific flurogenic hybridization probes to be designed and synthesized.

Magnetic immuno capture PCR assay (MIPA) for the detection of *L.borgpetersenni* serovar *hardjo* was developed (Taylor *et al.*, 1997). The immunomagnetic separation of leptospirosis from inhibitors in frozen formalin fixed bovine urine prior to PCR detection resulted in a marked improvement on previous detection methods. MIPA was a rapid 5-step protocol requiring 70 minutes preparation time prior to amplification, which consistently detected 10 organisms.

Romero *et al.* (1998) detected leptospiral DNA in patients with aseptic meningitis by PCR. The results were compared with those of the MAT and ELISA for detection of IgM. 39.8% were positive by PCR whereas 8.74% and 3.88% were positive by MAT and ELISA-IgM, respectively.

Heinemann *et al.* (2000) detected and differentiated *Leptospira* serovars in bovine semen by PCR and RFLP. The results showed that all serovars were amplified and the detection threshold in semen samples of a bull was 100 bacteria/ml. Using restriction enzymes they could classify the 26 serovars into eight groups.

Barocchi *et al.* (2001) identified a new repetitive DNA element in an isolate of *L.interrogans* serovar *copenhageni* from a patient in Salvador, Brazil. They constructed a *Sau 3A* genomic library from this strain and screened for repetitive DNA elements. An insert of 438 bp (Rep 1) from one library clone hybridized to multiple chromosomal DNA fragments resolved electrophoretically after digestion with *BamHI, HindIII* and *MfeI.* A single oligonucleotide primer, designated iRep I was designed to generate multiple PCR amplicons of various electrophoretic mobility in a PCR typing method. The method distinguished the strains belonging to the eight pathogenic and three saprophytic species of the genus *Leptospira.* Although the iRep1 primer was unable to discriminate strains among *L.interrogans* serovar *copenhageni* isolates, it was able to differentiate strains belonging to different species and serogroups of *Leptospira* identified in Salvador. This PCR-based method provided a faster and less expensive alternative to serologic tests used in reference laboratories.

Thirty-five leptospiral serovars from the species *Leptospira interrogans, Leptospira borgpetersenii, Leptospira santarosai, Leptospira kirschneri, Leptospira weilii, Leptospira biflexa* and *Leptospira meyeri* were characterized by the low stringency single specific primer PCR (LSSP-PCR)

technique (Oliveira *et al.* 2003). All genomic fragments amplified with G1 and G2 primers from distinct serovars of *Leptospira* were 285 bp in length, with nucleotide variation observed most frequently among different genomic species.

2.5 Cloning And Expression Of Recombinant Proteins

Leptospiral antigens are of interest as potential virulence factors and as candidate serodiagnostic and immunoprotective reagents. Because of the deficiencies of LPS-based vaccines, protein components of the leptospiral outer membrane represented an important approach to the development of alternative immunoprotection strategies. Studies suggested that the leptospiral outer membrane (OM) had a relatively complex protein profile (Brown *et al.*, 1991; Haake *et al.*, 1991). Only a few leptospiral OM protein (OMPs) had been characterized in detail, including a porin, Omp L1 (Haake *et al.* 1993), and two lipoproteins Lip L36 (Haake *et al.*, 1998) and Lip L41 (Shang *et al.*, 1996). The most prominent band in the leptospiral total protein profile has been a protein with a molecular mass of approximately 32 kDa. Many of the most abundant proteins in spirochaetes are lipoproteins, including TpN47 of *Treponema pallidum* (Chamberlain *et al.* 1989), Osp A of *Borrelia burgdorferi* (Bergstrom *et al.*, 1989) the *Vmp* proteins of the relapsing Borrelia fever (Burman *et al.*, 1990) and *smpA* of *Brachyspira hyodysenteriae* (Thomas and Sellwood, 1993).

You *et al.* (1999) constructed a vector with pUC 18 backbone, cytomegalovirus IE1 enhancers, promoter and intron A transcription regulatory elements and the BGH polyadenylation sequences driving the expression of

leptospiral endoflagellar gene, *fla B2*. The recombinant plasmid when injected into NZ white rabbits resulted in generation of specific leptospiral antibody with very high ELISA titres. The result suggested that the technique of DNA injection should have an advantage over previous vaccine technologies.

Haake *et al.* (1999) examined the synergestic immunoprotective capacity of the leptospiral porin Omp L1 and OMP Lip L41 in Golden Syrian hamster model of leptospirosis. Active immunization of hamsters with *E.coli* membrane fractions containing a combination of Omp1-M and Lip L41-M was found to provide significant protection against homologous challenge with *Leptospira kirschneri* serovar *grippotyphosa.*

Haake *et al.* (2000) reported the cloning of the gene encoding the 32 kDa lipoprotein, designated Lip L32, the most prominent protein in the leptospiral protein profile. They obtained the N-terminal amino acid sequence of a staphylococcal V8 proteolytic-digest fragment to design an oligonucleotide probe. A Lambda-Zap I library containing *EcoRI* fragments of *Leptospira kirschneri* DNA was screened, and a 5.0 kb DNA fragment which contained the entire structural Lip L32 was identified. Lip L32 is lipid modified in a manner similar to that of other prokaryotic lipoproteins. The deduced amino acid sequence of Lip L32 would encode a 272 amino acid polypeptide with a 19 amino acid of signal peptide, followed by a lipoprotein signal peptidase cleavage site. Lip L32 was completely solubilized by Triton X-114 extraction of *Leptospira kirschneri*, phase separation resulted in partitioning of Lip L32 exclusively into the hydrophobic, detergent phase, which indicated that it is a

component of the leptospiral outer membrane. It has been found that Lip L32 is expressed not only during cultivation but also during mammalian infection. Immunohistochemistry demonstrated intense Lip L32 reactivity with *Leptospira kirschneri* infecting proximal tubules of hamster kidneys. They reported that Lip L32 as a prominent immunogen during human leptospirosis. The sequence and expression is highly conserved among pathogenic *Leptospira* species. These findings indicated that LipL 32 may be important in the pathogenesis, diagnosis and prevention of leptospirosis.

There is an urgent need for new vaccine strategies against leptospirosis. Branger *et al.* (2001) studied the 31-34 kDa protein fraction of *Leptospira interrogans* serovar *autumnalis* which conferred cross protection in a gerbil model of leptospirosis. In this study, N-terminal sequencing of a 32-kDa fraction and southern blotting of genomic DNA with corresponding degenerated oligonucleotide probes identified two of its constituents: hemolysis-associated protein I (Hap I) and the outer membrane protein I (Omp LI). These proteins were first expresend in *E.coli* to use them as pure immunogens in protective trials and as soluble antigens for ELISA. The recombinant Omp LI production yield was very low and rHap I produced in *E.coli* was tested in vaccination trials but showed no evidence of direct protection. Adenovirus mediated Hap I vaccination induced significant protection against a virulent heterologous leptospira challenge in gerbils, whereas a similar Omp I construct failed to protect the animals. These data indicated that Hap I or Lip L32 could be a good candidate for developing a new generation of vaccines and which could induce broad protection against leptospirosis.

Flannery *et al.* (2001) studied five recombinant leptospiral proteins: Lip L32, Lip L41, Hsp 58, Omp I and Lip L36. The PCR-amplified genes were ligated into the pRSET plasmid for expression as recombinant His 6 fusion protein. The Hsp 58 fragment was inserted into the pQE 30 expression vector and electroporated into *E.coli* M15 pREP4 cells. These expressed proteins were evaluated as antigens in ELISAs.

Matsunaga *et al.* (2002) identified leptospiral protein as antigens by screening a genomic expression library with serum from a rabbit hyperimmunized with formalin-killed, *Leptospira kirschneri* serovar *grippotyphosa*. Genes expressing known outer membrane lipoproteins Lip L32 and Lip L41, the heat shock protein Gro EL, and the α, ß, and ß' subunits of RNA polymerase were isolated from the library. A new leptospiral gene that in *E.coli* expressed a 45 kDa antigen with an amino-terminal signal peptide followed by the spirochetal lipobox Val_4-Phe_3-Asn_2-Ala_1-Cys_1 was isolated too. They designated this putative lipoprotein as Lip L45. Immunoblot analysis of a panel of leptospiral strains probed with Lip L45 antiserum demonstrated that many low-passage strains expressed Lip L45. In contrast, Lip L45 was not detected in high passage, culture attenuated strains, suggesting that Lip L45 is a virulence-associated protein. Moreover, all leptospiral strains tested, irrespective of culture passage, expressed a 31 kDa antigen that was recognized by LipL 45 antiserum. Southern blot and peptide mapping studies indicated that this 31 kDa antigen was derived from the carboxy terminus of Lip L45, therefore it was designated P31 Lip L45. Membrane fractionation studies demonstrated that P31 Lip L45 as a peripheral membrane protein. The level of

this protein was increased as Leptospira entered the stationary phase, which indicated that P31 Lip L45 levels was regulated. Experiments with hamsters indicated that Lip L45 was expressed during infection and indicated that Lip L45 was expressed by *Leptospira kirschneri* that colonized the renal tubule. These observations suggested that expression of Lip L45 responds to environmental cues, including those encountered during infection of a mammalian host.

Recombinant leptospiral OMPs could elicit immunity to leptospirosis in a hamster infection model (Cullen *et al.*, 2002). Previously characterized OMPs appeared highly conserved, and thus their potential to stimulate heterologous immunity was of critical importance. Outer membrane fractions were isolated from *Leptospira interrogans* serovar *lai* grown at 20,30 and 37°C with or without 10% fetal calf serum and, finally, in iron-depleted medium. The OMPs were separated by two dimensional gel electrophoresis. Gel patterns from each of the five conditions were compared via image analysis, and 37 gel-purified protein were tryptically digested and characterized by mass spectrometry (MS). Matrix-assisted laser desorption ionization time-of-flight MS was used to rapidly identify leptospiral OMPs present in sequence data bass. Lip L32, Lip L36, Lip L41 and Lip L48 were identified by this approach. The expression of Lip L36 was not apparent at temperatures above 30°C or under iron-depleted conditions. The Lip L32 was observed to undergo substantial cleavage under all conditions except iron depletion. Significant downregulation of these mass forms were observed under iron limitation at 30°C, but not at 30°C alone, which suggested that Lip L32 processing depended on iron-

regulated-extracellular proteases. Moreover under iron-depleted conditions there was no concomitant increase in the levels of the intact form of Lip L32. The temperature and iron-regulated expression of Lip L36 and the iron dependent cleavage of Lip L32 were confirmed by immunoblotting with specific antisera. Global analysis of the cellular location and expression of leptospiral protein will be useful in the annotation of genomic sequence data and in providing insight into the biology of *Leptospira*.

Recombinant antigen-based serologic tests have been widely used in screening for spirochaetal infections such as Lyme disease and Syphilies (Magnarelli *et al.,* 2000; Schmidt *et al.,* 2000). But the use of recombinant proteins for serodiagnosis of leptospirosis had not been widely investigated. Borrelial protein BBK 32 was evaluated as an antigen in the serodiagnosis of early and disseminated Lyme borreliosis (LB). BBK 32 was cloned and sequenced from eight isolates of the three pathogenic *Borrelia* species. By IgG Western Bloting or ELISA, upto 74 and 100% of acute and convalescent phase samples, respectively, from 23 patients with erythema migrans (EM) were positive for rBBK 32 protein from *Borrelia afzelii.* In total, 14 of 14 samples from patients with neuro borreliosis and 15 of 15 samples from patients with Lyme arthritis were positive. These findings indicated that the BBK 32 protein were promising serodiagnostic antigens for the detection of early and disseminated LB (Heikkila *et al.,* 2002).

Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Maintenance of leptospires

3.1.1.1 Leptospires

The reference leptospiral strains were maintained throughout the study which were obtained from Koninklijk Institute Voor De Tropen (KIT), Amsterdam, The Netherlands and National Leptospirosis Reference Centre, RMRC, Port Blair, India.

Serogroup	**Serovar**	**Strain**
Pathogenic leptospires *(Leptospira interrogans)*		
Autumnalis	*autumnalis*	Akiyami A
Canicola	*canicola*	Hond Utrecht IV
Grippotyphosa	*grippotyphosa*	Muskva V
Hebdomadis	*hebdomadis*	Hebdomadis
Icterohaemorrhagiae	*icterohaemorrhagiae*	RGA
Javanica	*poi*	Poi
Pomona	*pomona*	Pomona
Non-pathogenic leptospires *(Leptospira biflexa)*		
Andamana	*andamana*	CH11
Semeranga	*patoc*	Patoc I

3.1.1.2 Media

Ellinghausen McCullough Johnson and Harris (EMJH) base and EMJH enrichment media obtained from DIFCO Laboratories (USA) were used for culturing of leptospires.

3.1.1.3 EMJH liquid medium

EMJH liquid medium was prepared by dissolving 2.3 gm of EMJH base in 900 ml of triple glass distilled water. The pH was adjusted to 7.5. After autoclaving at 15 lb/inch^2 for 15 min, the medium was added with leptospiral enrichment at 10% level under sterile conditions. The medium was dispensed in 5 ml aliquot in screw capped tubes and used for culturing leptospires after the sterility test at 48-72 hours.

3.1.1.4 EMJH semisolid medium

EMJH semisolid medium was prepared by adding 0.2% bacteriological agar (Sigma) to EMJH basal medium and sterilized by autoclaving, then added enrichment at 10% level under sterile conditions. The medium was dispensed in 5 ml aliquots in screw-capped tubes aseptically and used for maintenance of the culture after the sterility test for 48 hours.

3.1.2 Reagents for DNA isolation

Tris - HCl stock solution (1M) pH 8.0

Tris base	12.11 g
Distilled water	80 ml

The pH was adjusted to 8.0 using concentrated HCl. Distilled water was added to make 100 ml. The solution was autoclaved.

EDTA stock solution (0.2 M) pH 8.0

EDTA, disodium salt	7.44 g
Distilled water	80 ml

The pH was adjusted to 8.0 with 10 N NaOH. Distilled water was added to make 100 ml. The solution was autoclaved.

Solution I

Tris - HCl, pH 7.6	10 mM
KCl	10 mM
$MgCl_2$	10 mM
EDTA, disodium salt	2 mM

Solution II

Tris - HCl, pH 7.6	10 mM
KCl	10 mM
$MgCl_2$	10 mM
EDTA, disodium salt	2 mM
NaCl	0.4 M

Lysozyme (5 mg/ml)

(Grade I from chicken egg white, Sigma)

Lysozyme, 5 mg was dissolved in 1 ml of triple distilled water. This was prepared fresh every time.

Sodium dodecylsulphate solution, 10%

NaCl, 5 M

Tris - EDTA (10 mM Tris, 1 mM EDTA), pH 8.0

Tris Acetate gel running buffer (TAE) (50 x)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA, disodium salt, $2H_2O$	37.2 g

The pH was adjusted to 8.5 and the volume was made upto 1 litre with distilled water.

Gel loading dye (6x)

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Ficoll (type 400)	25% in distilled water

The dye was stored at room temperature.

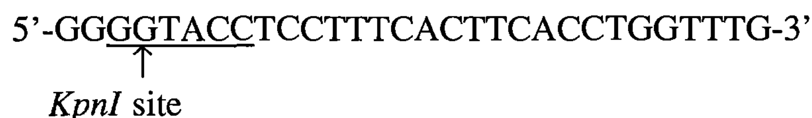
Ethidium bromide (2.5 mg/ml) (Sigma, USA)

3.1.3 Reagents for Polymerase chain reaction

Primers were designed using the OMEGA and Genetool softwares to amplify the partial Lip L32 gene of *Leptospira pomona*. The primers were also designed to have built in restriction enzyme sites to enable directional cloning of the PCR product into the expression vector.

Primer 1 (Forward)

Primer 2 (Reverse)



DyNAzyme ™II Recombinant enzyme kit (Finnzymes, Finland)

The kit contains the following reagents:

1. DyNAzyme ™II DNA Polymerase recombinant enzyme (2U/µl)
2. dNTP mix (10 mM each)
3. Optimized DyNAzyme II buffer (10 X).

Low Melting Point Agarose - GIBCO BRL, NY, USA.

DNA Extraction Kit (MBI Fermentas)

3.1.4 Molecular weight markers

λ DNA-HindIII and ϕX 174 DNA-*HaeIII* double digest mix (Finnzymes, Finland).

100 bp DNA ladder (MBI Fermentas).

3.1.5 Reagents for plasmid extraction

Solution I

Glucose	50 mM
Tris - HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

After autoclaving, the solution was stored at 4°C.

Solution II

Stock solutions of the following were prepared

Sodium Hydroxide (NaOH)	4 M
Sodium dodecylsulphate (SDS)	10%

200 mM NaOH and 1% SDS were freshly prepared from the stock solution just before use.

Solution III

Potassium acetate (5 M)	60 ml
Glacial acetic acid	11.5 ml
Triple distilled water upto	28.5 ml

To 5 M potassium acetate solution the required quantity of glacial acetic acid was added and pH then adjusted to 4.8 and volume made upto 100 ml. The resultant solution is 3 M with respect to adjusted potassium and 5 M with respect to acetate.

Phenol, Chloroform and Isoamyl alcohol (25:24:1) (Sigma, USA)

Isopropanol (Qualigens)

70% ethanol

3.1.6 Reagents for restriction enzyme digestion

Restriction enzymes *Sma*I, *Eco*RI, *Hind*III along with respective buffers were obtained from Gibco BRL, USA. *Xba*I and *Kpn*I along with the buffers were obtained from Genecraft, Germany.

3.1.7 Reagents for cloning and *in vitro* expression of Lip L32 gene

Cloning vector - pBluescript II KS (+) (Stratagene, USA).

Expression vector - pPro Ex HT 'a' (Life Technologies, USA).

Modifying enzymes

T_4 DNA Ligase (5 U/µl) and T_4 DNA Polymerase (5 U/µl) were obtained from MBI Fermentas.

3.1.8 Reagents for transformation and recombinant selection

***Escherichia coli* DH5α cells (Gibco BRL, USA)**

2M Mg^{++}

2 M solution was made containing 1 M each of $MgCl_2$ and $MgSO_4$ and sterilized.

SOB medium

Bacto-tryptone 20g, bacto-yeast extract 5 g, NaCl 0.5 g, KCl 0.186 g was dissolved in 950 ml triple distilled water. The pH 7.0 adjusted with NaOH and made the volume upto 1000 ml before autoclaving. When cool, 10 ml of sterile 2 M Mg^{++} solution was added.

Glucose, 20 mM was prepared, sterilised and added to SOB medium to make SOC.

Luria - Bertani (LB) Broth - Hi Media

LB Agar (2 g per 100 ml) - Hi Media

Ampicillin stock solution

Ampicillin (Sigma, USA), 50 mg was dissolved in 1 ml of triple distilled water and sterilized by filtration through a 0.22 micron filter. The stock solution was stored at -20°C.

LB - Ampicillin plates

LB agar (250 ml) was melted for about 25 min at medium power in a microwave oven. Allowed to cool for few minutes at room temperature and transferred to a water bath at the range of 45 - 50°C for about 30 min to equilibrate temperature. 250 μl volume of 50 mg/ml ampicillin was added. Temperature above 55°C will inactivate ampicillin. For "blue - white" selection

250 µl IPTG (20 mg/ml) and 250 µl X-gal (20 mg/ml) were added, mixed by swirling gently to avoid bubbles and poured on plates (approx 30 ml/plate). The plates were cooled and dried under sterile airflow. The plates were stored at 4°C for a month or less.

IPTG stock solution (Bangalore Genei)

Isopropylthio-ß-D-galactoside (IPTG) was dissolved at a concentration of 20 mg/ml in distilled water and sterilized by filtration through 0.22 µm filter and stored at -20°C.

X-gal stock solution (Bangalore Genei)

5-Bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) at 20 mg/ml in dimethylformamide was dissolved and stored in aliquotes at -20°C. The tube was wrapped in aluminium foil to prevent damage by light.

3.1.9 Reagents for preparation of competent cells

Ice cold Calcium Chloride (Sigma, USA), 50 mM.

Ice cold Glycerol (Sigma, USA), 50%.

3.1.10 Reagents and equipments for automated nucleotide sequencing of the cloned Lip L32 gene

Sequencing kit - Perkin Elmer Inc, USA ABI prism™ 3700 automated.

Capillary sequencer - PE - Big Dye® Terminator Cyclo Sequencing Kit, Applied Biosystems, USA.

3.1.10.1 Softwares for automated sequencing

ABI prism™ sequence data collection software - PE Applied Biosystems, USA.

ABI prism™ auto assembler software - PE Applied Biosystem, USA.

3.1.10.2 Softwares for Phylogenetic analysis

CLUSTAL X Version 1.7 Documentation

Multiple sequence alignment programme (Thompson *et al.,* 1994) available at web site ftp/ebi.ac.uk/pub/software.

DNASTAR

Meg Align package in Lasergene obtained from DNASTAR, Inc., Madison, USA.

MEGA (Molecular Evolutionary Genetic Analysis) version 1.02

3.1.10.3 Software for open reading frame and protein sequence determination

GeneTool Lite from http://www/Double Twist.com and OMEGA were used for finding the open reading frames and conceptual protein sequence determination by translation of nucleotide sequence.

3.1.11 Reagents for purification of recombinant Lip L32 protein

Binding Buffer (Buffer A)

NaCl	0.50 M
Tris	0.10 M
NaH_2PO_4	0.05 M
Urea	8.00 M
pH	7.5

Washing Buffer (Buffer B)

NaCl	0.50 M
Tris	0.10 M
$NaH_2 PO_4$	0.05 M
Urea	8.00 M
pH	6.5

Elution Buffer (Buffer C)

NaCl	0.50 M
Tris	0.10 M
$NaH_2 PO_4$	0.05 M
Urea	8.00 M
Imidazole	0-750 mM
pH	6.5

Chelating Sepharose Fast Flow (Amersham, U.K.) International PLC.

Nickel chloride, hexahydrate, extrapure (Himedia)

50 mM EDTA

Dialysis bag which retain proteins with M.W. > 12,000 (Sigma, USA)

Phosphate bufferred saline (PBS) containing 10% (vol/vol) glycerol

Total protein and albumin kit (Dr.Reddy's Laboratories, India)

3.1.12 Reagents for characterisation of recombinant Lip L32 protein

3.1.12.1 Reagents for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide stock solution

Acrylamide, AR	30.0 g
N,N'-Methylene bis-acrylamide, AR	0.8 g

Distilled water was added to make 100 ml. The solution was filtered and stored at 4°C.

Separation gel buffer (1.5 M Tris - HCl, pH 8.8)

Stacking gel buffer (0.5 M Tris - HCl, pH 6.8)

Sodium dodecylsulphate 10% stock solution

TEMED (Gibco BRL, USA)

Ammonium persulphate (SRL, India)

Destaining Solution I

Methanol	50%
Acetic acid	10%
Distilled water	40%

Electrophoresis buffer

Tris	25 mM
Glycine	250 mM
SDS	0.1%
The pH adjusted to 8.3	

2X SDS sample buffer

Tris - HCl (pH 6.8)	125 mM
SDS	4%
Glycerol	20%
Bromophenol	0.01%

Coomassie Brilliant blue stain

0.25 g Coomassie Brilliant Blue R 250 was dissolved in 90 ml of methanol and water (1 : 1 v/v) and 10 ml of glacial acetic acid. The solution was filtered through a Whatman no.1 filter.

Destaining solution II

Glacial acetic acid	10%
Glycerol	7%
Distilled water	83%

3.1.12.2 Reagents for Western Blotting

Hybond™ C, Nitrocellulose membrane (NCM)
(Amersham International PLC, UK)

Phosphate Bufferred Saline - Tween 20 washing solution

Sodium chloride	8.0 g
Potassium dihydrogen phosphate	0.2 g
Di-sodium hydrogen phosphate	2.9 g
Potassium Chloride	0.2 g
Tween - 20	0.5 ml

Distilled water was added to make upto 1 litre

Bovine serum albumin (BSA) - Fraction V (Sigma, USA)

Hyperimmune mice serum against *Leptospira pomona*

Hyperimmune rabbit serum against *Leptospira patoc* obtained from Leptospirosis Research Laboratory, Madhavaram, Chennai.

Goat anti-mouse HRP conjugate (Bangalore, Genei)

Goat anti-rabbit HRP conjugate (Bangalore, Genei)

Colour indicator Solution

Diamino benzidine (DAB) 50 mg was dissolved in 100 ml of PBS, pH 7.2 and stored at 4°C.

Colour indicator working solution

Colour indicator stock, 3 ml was mixed in 9 ml of PBS, then 150 μl one percent H_20_2 was added immediately before adding to NCM.

3.1.13 Animals and reagents for production of hyperimmune serum

White mice

Male white mice, 6-8 weeks old obtained from Laboratory Animal Medicine, Madhavaram were used for raising hyperimmune serum.

Antigen for immunization

Pure cultures of *Leptospira pomona* in EMJH liquid medium were used for immunization.

3.1.14 Reagents required for preparation of *Escherichia coli* Lysate

Resuspension buffer

Tris HCl (pH 8.0)	50 mM
EDTA (pH 8.0)	10 mM

The buffer was filter sterilized and stored in 50 ml aliquot at 4°C.

Blocking buffer

Tris - HCl (pH 8.0)	10 mM
NaCl	150 mM
Tween 20 v/v	0.05%
BSA	3%

Blocking buffer was stored at 4°C and can be used several times when sodium azide was added to a final concentration of 0.05% (w/v) to inhibit the growth of microorganisms.

3.1.15 Reagents for Enzyme linked immunosorbent assay (ELISA)

Recombinant Lip L32 antigen

Rabbit anti-human and anti-dog IgG - HRP conjugate (Bangalore Genei)

Human and dog serum samples

Carbonate-bicarbonate buffer (pH 9.6)

Sodium Carbonate	1.59 g
Sodium bicarbonate	2.93 g

Distilled water was added, pH was adjusted to 9.6 and volume was made upto 1 litre.

Sodium citrate buffer (pH 4.2)

Sodium citrate 14.705 g

Distilled water was added, pH was adjusted to 4.2 and volume was made upto 1 litre.

2-2'-Azino di-ethylbenz-thiazoline-6-sulphonic acid (ABTS) Substrate Sigma, USA

The other components such as phosphate buffered saline Tween 20 washing solution and BSA were as described in section 3.1.9.4.

3.1.16 Reagents for Dipstick ELISA

Nitrocellulose membrane 0.45 μ (Sigma, USA)
Skimmed milk powder (Amul)

Other components such as recombinant Lip L32 antigen, rabbit anti-dog and anti-human IgG-HRP conjugates, substrate, serum samples, PBST, carbonate - bicarbonate buffer, sodium citrate buffer were as described in section 3.1.13.

3.1.17 Reagents for Latex agglutination test

Latex beads (0.8 μ) (Sigma, USA)
Sodium azide (NaN_3) - Analytical Reagent (CDH, Bombay)

The other components were BSA, PBS, carbonate - bicarbonate buffer and serum samples suspected for leptospiral infection.

3.1.18 Reagents for Microscopic agglutination test

96-well, U bottom microtitre plates (Nunc, USA) were used for conducting MAT

Antigen - MAT was performed using live cultures from serovars *pomona, icterohaemorrhagiae, canicola, grippotyphosa, autumnalis, javanica* and *hebdomadis.*

3.2 Methods

3.2.1 Culturing of leptospires

Stock cultures were maintained in semisolid media in screwcapped tubes and stored in dark at room temperature. Stock cultures were routinely subcultured at 8 weeks interval and incubated at room temperature. The purity of culture was examined under dark field microscope. The liquid EMJH media was used for culturing the leptospiral organisms for DNA extraction and PCR.

3.2.2 DNA isolation

The DNA was extracted as per the method of Lahiri and Nurunberger (1991) and Senthilkumar and Ramadass (2001) with minor modifications.

i. Five ml of 7-10 days old leptospiral cultures (approximately 5×10^8 organisms/ml) were centrifuged at 10,000 rpm for 20 min to pellet the organisms.

ii. The pellet was washed twice with solution I.

iii. The final pellet was resuspended in 0.5 ml of solution II.

iv. Freshly prepared lysozyme (5 mg/ml) solution, 50 µl was added and kept at 37°C for 15 min.

v. Fifty µl of 10% Sodium dodecyl sulphate, was added and mixed by pipetting.

vi. Sodium chloride (5 M) solution, 250 µl was added and mixed by pipetting.

vii. The mixture was centrifuged at 10,000 rpm for 5 min.

viii. The supernatant was collected in a fresh microfuge tube and 2 volumes of absolute alcohol was added at room temperature.

ix. Then the tube was mixed by gently inverting and centrifuged at 10,000 rpm for 5 min at 4°C.

x. The DNA pellet was washed once with 70% ethanol and the pellet was air dried.

xi. The dried pellet was resuspended in TE (10 mM Tris, 1 mM EDTA).

The purity and concentration of the DNA was checked by 0.8% agarose gel electrophoresis and UV-spectrophotometer, respectively.

3.2.3 Polymerase chain reaction for amplification of partial Lip L32 gene

3.2.3.1 Designing of primers

All the sequences of the *Leptospira* serovars available in GenBank were downloaded from the website http://www.ncbi. nlm.nih.gov/ entrez. The Lip L32 sequence of available *Leptospira* serovars were selected and converted into FASTA format and stored in a single file. These sequences were loaded into the Clustal programme and aligned to find out the homology regions. Sequences corresponding to the areas 130 to 150 and 757 to 778 of the *Leptospira kirschneri* Lip L32 nucleotide sequence were selected as primers with restriction sites added to the primer for directional cloning.

3.2.3.2 Polymerase chain reaction (PCR)

The PCR for the Lip L32 gene (approxiomately 665 bp including the restriction sites) was carried out in a 50 μl reaction.

PCR mix for 50 μl volume :

10X PCR buffer	5.0 μl
10mM dNTP mix	0.5 μl
Forward primer (10 mM)	0.5 μl
Reverse primer (10 mM)	0.5 μl
Taq DNA Polymerase (5 U/μl)	0.5 μl
DNA	1.0 μl
Nuclease free water	42.0 μl

PCR Programme

The amplification was carried out with an initial denaturation at 94°C for 5 min followed by a step up PCR as per the conditions given below:

94°C for 1 min	
57°C for 1 min 30 seconds	25 cycles
72°C for 2 min	

A final extension hold at 72°C for 10 min was given followed by ramping to 4°C. Ten μl of the amplified product was checked in a 1.2% agarose gel with DNA molecular weight markers.

3.2.3.3 Purification of the amplified Lip L32 gene product

Purification of the PCR product was carried out as per the manufacturers protocol in DNA extraction kit (MBI, Fermentas).

The amplified Lip L32 gene (approx 665 bp) was electrophoresed in a 1.2% Low melting point agarose gel and the band was excised from the gel. The gel band was placed in a fresh eppendorf and the weight of the eppendorf was determined before and after placing the gel piece. Three volumes of binding solution was added to one volume of gel and incubated at 55°C for 5 minutes to dissolve the agarose. Six µl of the resuspended silica powder suspension was added to the eppendorf and incubated for 5 minutes at 55°C. Mixed by vortexing every 2 min to keep silica powder in suspension. The silica DNA complex was centrifuged at 10,000 rpm for 2 minutes to form a pellet and the supernatant was discarded. Five-hundred µl of ice cold wash buffer was added, vortexed and centrifuged at 10,000 rpm for 2 min and the supernatant was poured off completely. The pellet was air - dried for 10-15 min. The DNA was eluted into water or TE buffer. The pellet in an aliquot of water or TE was resuspended and the tube was incubated for 5 min at 55°C. The tube was centrifuged for 2 min at 10,000 rpm and the supernatant was immediately transferred to a new tube avoiding the pellet. The concentration of DNA was determined by running an aliquot on 1.2% agarose gel.

3.2.4 Cloning of the Lip L32 gene into pBluescript II KS (+) vector

The pBluescript II KS (+) phagemid is a 2961 bp derived from pUC19. The vector has a multiple cloning site flanked by T3 and T7 RNA promoters and an Ampicillin resistance gene for antibiotic selection of the phagemid vector. The protocol of Sambrook and Russel (2001) was followed with some modifications.

3.2.4.1 Restriction enzyme digestion of pBluescript II KS (+)

The pBluescript II KS (+) phagemid was made blunt end by digesting with *Sma*I restriction enzyme.

3.2.4.2 Filling up of Lip L32 amplicon with T4 DNA Polymerase

The PCR product of Lip L32 was made blunt end by T4 DNA Polymerase. The reaction was set up as described below :

Lip L32 gene (amplified gene)	15 µl
5X T4 DNA polymerase buffer	8 µl
dNTP mix (10 mM)	2 µl
T4 DNA polymerase (5 U/µl)	1 µl
Nuclease free water made upto 40 µl	

The reaction components were incubated at 11°C for 15 min. The enzyme was denatured at 65°C for 20 min.

3.2.4.3 Ligation of pBluescript II KS (+) vector and Lip L32 gene

To clone the outer membrane protein gene Lip L32 into the pBluescript II KS (+) phagemid vector, a ratio of 1:3 of vector to insert was used. The ligation reaction was set up as described below:

pBluescript II KS (+) vector	1.5 µl
Insert	2.0 µl
10X T4 DNA Ligase Buffer	1.0 µl
T4 DNA Ligase (5 U/µl)	1.0 µl
PEG 4000	1.0 µl
Nuclease free water made upto 10 µl	

The mixture was incubated overnight in a water bath at 16°C for ligation and an aliquot of the mixture was used for transformation.

3.2.4.4 Competent cell preparation

Escherichia coli DH5α cells were streaked onto LB plate from the stock and incubated at 37°C overnight. A single colony was picked from the LB plate and inoculated into 5 ml of LB broth. The broth was incubated at 37°C overnight. The next day 200 μl of the overnight grown culture was inoculated into 200 ml of LB broth and incubated with the rotary shaking speed of 200 rpm. When the optical density (OD) reaches 0.4 at A_{600} the growth was arrested by chilling on ice. The flask was chilled for 10 minutes. The culture was centrifuged at 5000 g for 10 min at 4°C. The supernatant was discarded. Keeping the tube on ice, the bacterial pellet was resuspended in 30 ml of ice-cold 50 mM $CaCl_2$. The cells were kept on ice for 25 min and centrifuged at 5000 g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml of ice cold 50 mM $CaCl_2$ containing 15% glycerol (resuspension should be done very gently as the cells are very fragile at this stage). The suspension in 100 μl volume was aliquoted in cold microcentrifuge tubes and stored at -70°C.

3.2.4.5 Transformation and recombinant selection

1. LB Ampicillin/IPTG/X-gal plates were prepared prior to the transformation and were equilibrated at room temperature prior to plating.
2. 100 μl aliquot of competent DH5α cells were allowed to thaw leaving the tube on ice.
3. When thawed completely, 5 μl of the ligation mixture was transferred to the tube containing competent cells.

4. The tube was gently flicked to mix and left on ice for 40 min.
5. The cells were subjected to a heat shock at 42°C for 50 sec and immediately returned to ice for 1-2 min.
6. SOC medium, 800 µl was added to the cells and incubated for 50 min at 37°C with shaking.
7. The cells were spread onto LB agar plate with Ampicillin/ X-gal/IPTG and incubated at 37°C overnight for selection of the recombinant clone.

3.2.4.6 Colony PCR

The white colonies which were supposed to be recombinant clones were picked from the LB ampicillin plate and streaked onto another LB Ampicillin plate and grown overnight at 37°C. Next day, colony PCR was performed using T3 primer specific for pBluescript II KS (+) vector and reverse gene specific primer. Few cells from the LB Ampicillin plate were mixed with the PCR components and PCR and carried out as in Section 3.2.3.2

3.2.4.7 Miniprep plasmid isolation

Recombinant plasmid (pKSP4) was extracted from the positive colonies as detected in colony PCR.

1. From overnight cultures of the colonies in LB broth, 1.5 ml was transferred into 2 ml microfuge tubes.
2. The tubes were centrifuged for 5 min at 8,000 rpm and the medium was completely aspirated.

3. Ice cold solution I, 100 µl was added and the cells were completely resuspended by vigorous vortexing.
4. Freshly prepared solution II, 200 µl was added and the tubes were inverted 4-6 times till the tube turns somewhat clear and viscous and incubated on ice for 5 min.
5. Ice cold solution III, 150 µl was added and mixed by inversion.
6. After 10 min incubation on ice, the tubes were centrifuged at 12,000 rpm for 5 min in a microfuge.
7. The supernatant was carefully transferred into a fresh tube and equal volume of Phenol-Chloroform-Isomyl alcohol (25:24:1) was added to the tube.
8. The tubes were centrifuged at 12,000 rpm for 5 min and the aqueous layer was collected in a fresh tube.
9. Isopropanol, 0.8 volume was added to the tube and mixed well.
10. The tubes were centrifuged at 12,000 for 15 min and the supernatant discarded carefully.
11. The pellet was washed with 0.5 ml of 70 per cent ethanol and air dried.
12. The pellet was resuspended in 20 µl of TE buffer (pH 8.0) or nuclease free water and RNase (10 mg/ml) and incubated at 37°C for 1 hour.
13. The plasmids were electrophoresed in a 1 per cent agarose gel in TAE buffer.

3.2.4.8 Restriction enzyme digestion of the recombinant plasmid

The plasmid extracted from the white colonies was subjected to digestion with the restriction enzymes viz., *Eco*RI, *Xba*I, *Kpn*I and *Sac*I to check the presence of the insert as well as to ascertain the orientation of the insert.

3.2.5 Automated sequencing of the cloned Lip L32 gene

The recombinant plasmid pKSP4 was purified with DNA extraction kit as per manufacturer's (MBI, Fermentas) protocol and given for sequencing to M.S.Swaminathan Research Foundation, Chennai.

3.2.5.1 Phylogenetic analysis of the obtained Lip L32 gene sequence by nucleotide sequence analysis

The Lip L32 sequence of the serovars of Leptospira available as on date in GenBank were written onto a single text file in FASTA format and used as input file for the programme CLUSTAL X 1.8. Meg Align package was used to align all the sequences in the worktable using the Clustal V method developed by Higgins and Sharp (1989). The Clustal method groups sequences into clusters by examining sequence distances between all pairs. Clusters are aligned as pairs, then collectively as sequence groups, to produce the overall alignment. After the multiple alignment is completed, a Neighbour-Joining method (Saitou and Nei, 1987) was employed to reconstruct phylogeny for the putative alignment.

Molecular Evolution and Genetic Analysis (MEGA) software V 1.02 was used to find out the varying amino acids between the serovars.

3.2.5.2 Open reading frame (ORF) and protein sequence estimation

The GeneTool Lite programme was used to find out the open reading frames of the nucleotide sequence data obtained in all three reading frames. The reading frame without any stop codons and giving a full-length protein was conceptually translated into protein sequence using the same software package.

3.2.6 *In vitro* expression of Lip L32 gene

The protocol from Sambrook and Russel (2001) was followed with minor modifications.

3.2.6.1 Preparation of the expression vector

Purified pPro EX HT (a) plasmid DNA 1 µg was digested with the restriction enzymes viz., *Xba*I and *Kpn*I to generate complementary overhangs. The linearized plasmid DNA was electrophoresed in 1% Low Melting Point (LMP) Agarose gel in TAE buffer and the linear vector was eluted from the gel as described in section 3.2.4.3

3.2.6.2 Preparation of the insert

The recombinant plasmid pKSP4 was digested with *Xba*I and *Kpn*I to release the insert (approximately 665 bp) with complementary overhangs. The insert was visualized in 1.2% LMP Agarose and was eluted from the gel as described in section 3.2.3.3.

3.2.6.3 Ligation and transformation

The directional cloning of the outer membrane protein Lip L32 gene into the expression vector pPro EX HT 'a' was set up as described in section 3.2.4.3. The ligation mixture was transformed in *Escherichia coli* DH5α cells as described in Section 3.2.4.5.

3.2.7 Recombinant selection

3.2.7.1 Colony PCR

The recombinant clones were picked from the LB Ampicillin plate and streaked onto another LB Ampicillin plate and grown overnight at 37°C. Colony PCR was performed using M13 reverse sequencing primer specific for the vector and reverse gene specific primer. PCR was carried out as described in section 3.2.3.2.

3.2.7.2 Restriction enzyme digestion of the recombinant plasmid pPro10

The recombinant plasmid pPro10 extracted from the recombinant colony (described in Section 3.2.4.7) was subjected to digestion with the following restriction enzymes viz., *Eco*RI, *Hind*III, *Xba*I, *Kpn*I to check the presence of the insert.

3.2.7.3 Induction of Lip L32 recombinant protein

1. A single recombinant colony (pPro10) was picked from the LB Ampicillin plate and innoculated into 5 ml of LB Ampicillin broth. The broth was incubated overnight at 37°C with the rotary shaking speed of 200 rpm.

2. The next day, 0.1 ml of overnight culture was inoculated into 10 ml of LB Ampicillin broth and grown at 37°C with agitation.

3. When the culture reached an A_{590} of 0.5-1.0, 1 ml was removed and centrifuged for 1 min in a microcentrifuge. The supernatant was discarded and the cells were resuspended in 100 μl of PBS. This was the uninduced sample.

4. To the remaining culture, IPTG (1 mM) was added and continued to incubate the culture as described above.

5. One ml aliquots of cells were removed, 1, 2, 3 and 4 hours post-induction and measured the A_{590}. The cells were centrifuged as above and the pellets were resuspended in 100 μl of PBS. These were the induced samples.

3.2.7.4 Purification of Lip L32 recombinant protein

Growth of Expression Culture

1. LB Ampicillin broth, 10 ml was inoculated with a single recombinant colony (pPro10) and incubated at 37°C with agitation.

2. The next morning, 1 ml of the overnight culture was inoculated into 100 ml of LB Ampicillin broth and incubated at 37°C with agitation.

3. When the culture reached an A_{590} of 0.5-1.0, a sample prior to induction was removed to serve as an uninduced control. The remaining culture was induced with IPTG (1 mM) and continued to incubate the culture at 37°C for 3 hours (optimal induction time as determined in section 3.2.6.5).

4. At the end of the induction period the cells were centrifuged at 10,000g for 10 min. The supernatant fluid was decanted and the pellet stored at -70°C until ready for protein purification.

Preparation of cleared Lysates under denaturing conditions

1. The cell pellet was thawed for 15 min on ice and resuspended in 10 ml of Buffer A (Binding Buffer).

2. The cells were lysed by gentle vortexing and kept on the racker in the cold room overnight.

3. The next day, the lysate was centrifuged at 10,000g for 30 min at 4°C to pellet the cellular debris.

Purification under denaturating conditions

1. Nickel chloride (600 mg per 10 ml) solution, 3 ml was loaded into a column containing 1 ml of sepharose and kept for 15 min until the sepharose turns blue.

2. The bottom cap was removed and the column was washed with 10 column volumes (cv ie 10 x 1 ml) of water.

3. The column was equilibrated with 11 cv of Buffer A.

4. 10 ml of cleared lysate was applied to the column and the flow through was collected.

5. The column was washed with 20 cv of wash buffer (Buffer B) until the flow through at A_{280} is less than 0.01.

6. The protein was eluted with a 40 ml gradient of 0-750 mM imidazole in wash buffer (Buffer C). Each fractions were collected and analyzed on SDS-PAGE.

7. Finally, the column was regenerated using 10 cv of 50 mM of EDTA.

3.2.7.5 Dialysis of the purified recombinant Lip L32 protein

The dialysis tube (25 mm) was prepared as per the standard procedure (Sambrook and Russel, 2001). Recombinant protein was dialyzed overnight at 4°C on a magnetic stirrer against phosphate buffered saline (PBS) containing 10% (vol/vol) glycerol and 0.025% (wt/vol) sodium azide.

3.2.8 Characterization of Lip L32 recombinant antigen

3.2.8.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Recombinant Lip L32 antigen

Uninduced and induced expression cultures, 15 µl each, were taken in a fresh microcentrifuge tube and mixed with an equal volume of 2X SDS sample buffer. The samples were boiled and used for SDS-PAGE.

Each fractions collected during purification of recombinant Lip L32 protein were boiled and mixed with an equal volume of 2X SDS sample buffer. The samples were boiled and used for SDS-PAGE.

SDS-PAGE for proteins (Laemmli, 1970)

1. The gel for SDS-PAGE was made using glass plates with spacer bar of 0.75 mm thickness. The separating gel of 12% was prepared using the following components.

Distilled water	3.3 ml
30% Acrylamide mixture	4.0 ml
1.5 M Tris - HCl (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% Ammonium persulphate	0.1 ml
TEMED	0.004 ml.

2. Before addition of ammonium persulphate and TEMED, the mixture was degassed to remove any air bubbles.

3. The mixture was poured in between the glass sandwich. Small quantity of distilled water was added over the gel surface to have uniform surface and quick polymerization.

4. The stacking gel of 5% acrylamide was prepared with the following composition.

Distilled water	3.40 ml
30% Acrylamide mixture	0.83 ml
1.0 M Tris-Hcl (pH 6.8)	0.63 ml
10 % SDS	0.05 ml
10% Ammonium persulphate	0.05 ml
TEMED	0.005 ml

5. The comb was placed in between the glass sandwich and the stacking gel mixture was poured and allowed for complete polymerization.

6. The comb was removed, the wells were rinsed with Tris-glycine running buffer and the gel plate was attached to the electrophoresis apparatus.

7. The samples were loaded and electrophoresis was carried out at a constant volt of 100 until the bromophenol blue marker dye reached the bottom of the gel.

8. The glass sandwich was removed carefully, the stacking gel discarded, orientation made and the gel was transferred to the Coomassie Brilliant Blue staining solution.

9. Staining was carried out for 1 to 2 hours and then transferred to destaining solution I.

10. Once the gel background became clear, the gel was transferred and stored in destaining solution II.

3.2.8.2 Western blot technique

Preparation of Hyperimmune serum

Four numbers of six to eight weeks old white mice were immunized with live leptospires cultured in EMJH medium. All the mice were immunized twice with intraperitoneal injection of 0.5 ml live leptospires. The second and third injections were given 30 and 59 days after the first injection.

Removal of cross-reactive Antibodies from Hyperimmune serum

A 100 ml culture of *Escherichia coli* DH5α cells were grown to saturation in LB broth. The cells were harvested by centrifugation at 5500 rpm for 10 min at 4°C and then resuspended in 3 ml of cell suspension buffer. The cell suspension was frozen and thawed several times and then sonicated at full power for six periods of 20 seconds, each at 0°C. The extract was centrifuged at maximum speed for 10 min at 4°C in a microcentrifuge tube. The supernatant was transferred to a fresh tube and the lysate stored at - 20°C.

Just before using the lysate, the antiserum that is to be used for screening was diluted 1:10 with blocking buffer. 0.5 ml of processed lysate was added for every ml of antibody preparation to be used. The mixture was incubated at 4°C over night in the presence of 0.05% (w/v) sodium azide until used for immunological screening.

Immunoblotting

After SDS-PAGE, the proteins in the gel were transferred onto a nitrocellulose membrane in a semi-dry Biorad Western blot apparatus using western transfer buffer (Trisbase 2.9 g, glycine 1.45 g, 50 ml of methanol in a final volume of 500 ml deionized water). Two pieces of Whatman No.3 filter papers were soaked in transfer buffer and placed on the top and bottom of the stack. Before transfer, bubbles were carefully removed from the stack using a glass rod. Transfer was performed for 2 hrs at a constant current of 0.8 mA/cm^2 of the nitrocellulose membrane.

1. After the transfer, the NCM was washed briefly in water and incubated in blot buffer for one hour at 37°C and then washed with PBS-Tween for one hour at room temperature.

2. The NCM was saturated with 2% BSA in PBS (pH 7.5) for 2 hour to prevent non-specific protein binding.

3. NCM was incubated with diluted antiserum (1:200) overnight at 4°C.

4. The filter was washed in a shallow dish with four changes of 50 ml of PBST for a total of 200 ml over 30 min.

5. The goat anti-mouse - HRP was optimally diluted in PBST and 0.5 ml was added and incubated for one hour at room temperature with rocking.

6. After washing, the colour indicator solution was added at room temperature with rocking until there was a colour development.

7. The colour development was halted by washing with water. Then the developed NCM was dried and stored in dark.

3.2.8.3 Quantitative determination of the recombinant Lip L32 protein

Three test tubes were taken and labelled them as 'Blank' 'Standard' and 'Test' respectively. To each of the test tube 500 µl of Biuret reagent and one ml of distilled water was added. To the test tubes marked 'Standard' and 'Test', 25 µl of standard and recombinant Lip L32 protein were added respectively. All the reagents were mixed well and incubated at 37°C for 10 min. Finally, the absorbance of Standard (S) and Test (T) against blank (B) on spectrophotometers at 555 nm were measured. The concentration of the protein was calculated as

$$\text{Total protein in gm\%} = \frac{\text{A of (T)}}{\text{A of (S)}} \times \text{Std.Conc.}$$

3.2.9 Detection of leptospiral antibodies in clinical samples by recombinant Lip L32 antigen.

3.2.9.1 Enzyme linked immunosorbent assay (ELISA)

Preparation of ELISA antigen

Purified recombinant Lip L32 antigen was diluted varying from 5 ng to 1.5 µg per well in 0.06 M carbonate-bicarbonate buffer (pH 9.6). Each antigen dilution was coated in duplicate wells of the 96 well plates. The optimum concentration of coating antigen was determined by checkerboard titration in ELISA.

Standardization of rabbit anti-human IgG - HRP conjugate concentration

Dilution of conjugate in PBST was arrived by checkerboard titration. 1:4000 dilution was used.

Standardization of rabbit anti-dog IgG - HRP conjugate concentration

Dilution of conjugate in PBST was arrived by checkerboard titration. 1:3000 dilution was used.

Preparation of serum samples

A total 113 of canine and 136 human serum samples were screened. Human samples were kindly provided by Lister Laboratory, Chennai, Madras Medical College, Chennai, Leptospirosis Research Laboratory, Madhavaram, Chennai. Canine blood samples were collected from Madras Veterinary College Hospital, Chennai. Blood samples were collected taking asceptic precautions, allowed to clot and then centrifuged at 2000 rpm for 20 min for the separation of serum. Serum samples were stored at -20°C until use.

Preparation of ABTS substrate solution

Fifty five mg of ABTS (2,2'-Azino di-ethylbenz-thiazoline-6-sulphonic acid) was added to 25 ml of sodium citrate buffer and 25 µl of 35% hydrogen peroxide was added to it and immediately dispensed into the plates.

Indirect ELISA

The human and dog serum samples were diluted 1: 125 to 1: 16,000 and tested by ELISA. The rabbit anti-human and anti-dog IgG HRP conjugate concentrations were determined by checkerboard titration in ELISA. IgG - ELISA was performed as per the method of Adler *et al.* (1981) with some modifications.

1. Fifty μl of recombinant Lip L32 (rLip L32) antigen was diluted in 24.5 μl of carbonate bicarbonate buffer (pH 9.6) and used for coating 96-well polystyrene microtitre plates.

2. The plates were incubated at 4°C for overnight.

3. At the end of incubation, the plates were washed in PBST three times and the plates were stored at 4°C until use.

4. Before the addition of sera, the plates were washed once with PBST and 100 μl of 2% bovine serum albumin was added to each well to block unreacted sites in the wells and incubated for 1 hr at 37°C.

5. After incubation, the plates were washed three times in PBST and dried by taping against the filter paper.

6. Appropriate dilution of serum samples, positive and negative control sera (1: 125) were added in duplicate.

7. The plates were incubated for 1 hr at 37°C and then washed three times with PBST.

8. The diluted conjugate 100 μl were added to each well except the substrate control well. HRP control was also used in each plate.

9. The plates were incubated for 1 hr at 37°C. After incubation, the plates were washed three times with PBST.

10. The substrate solution in sodium citrate buffer and hydrogen peroxide was prepared and added immediately to all the wells in 100 μl volume.

11. The plates were incubated in dark for 10-15 min for the development of colour reaction.

12. The plates were read at 405 nm in ELISA reader (Bio-Tek Instruments Inc., USA). The OD value of substrate control well was blanked.

Measurement of Antibody Activity Titre in a Single Serum Dilution

ELISA antibody titres were determined by standard serial dilutions and end-points were calculated by a subtraction method (Snyder *et al.,* 1983) with some modifications

1. A total of 15 ELISAs were run with different negative serum pools to provide absorbance data for the construction of a positive - negative threshold (PNT) baseline which was used to determine observed ELISA antibody titers.

2. A mean absorbance and three standard deviation units above the mean absorbance were calculated from replicates of all trials at each negative serum dilution.

3. The three standard - deviation baseline-unit values were then plotted in the Logarithmic graph in Microsoft Excel and used as the PNT baseline for calculation of the observed leptospiral antibody titres.

4. The absorbance of these test sample dilutions were corrected by subtracting the appropriate average absorbance of internal negative control serum dilutions from the same trial as

$$\text{S/P ratio} = \frac{\text{Sample absorbance - Negative control absorbance}}{\text{Positive control absorbance - Negative control absorbance}}$$

5. The corrected average absorbances of the test samples were graphed and the observed antibody end point titres were defined as the point at which the plotted lines intersect the PNT.

Positive antisera were titrated serially using the ELISA and corrected as described above.

The coefficient of correlation (r) between the $\log_{10}$ end titre and the S/P ratio (at serum dilutions of 1:125 to 1:16,000) were calculated. The dilution of serum (1: 125 to 1 : 16,000) showing the maximum value of 'r' was chosen for the derivation of the regression equation ($y = a + bx$, where $y = \text{Log}_{10}$ antibody titre of the test serum , x = the S/P ratio of the test serum at the

chosen dilution, a = the constant and b = the regression coefficient) for predicting Log_{10} end titre by single dilution ELISA. After regression analysis, a standard curve was constructed with the titre values obtained from these antisera. The predicted titre at a single working dilution was plotted against the observed antiserum titre. Subsequently, predicted antibody activity titres were determined directly from the standard curve by solving the regression line equation (Snyder *et al.*, 1983).

Indirect IgM ELISA

A commercially available IgM ELISA kit (Pan Bio Pty Ltd. Brisbane, Australia) obtained from Lister Laboratory was used to determine IgM antibodies in the human serum samples with recombinant Lip L32 (rLip L32) as antigen. The method was followed as per the manufacturer's protocol. A positive sample was defined as having a sample calibrator absorbance ratio of ≥ 1.0, and a negative sample was defined as having a sample calibrator absorbance ratio of < 1.0.

3.2.9.2 Dipstick ELISA

Dipstick ELISA relies on the attachment of antigens to nitrocellulose membranes. Dipstick ELISA was carried out as per the methods of Nielsen *et al.*, (1985) and Ramadass *et al.*, (1993) with minor modifications.

1. The dipsticks were prepared by pasting 20 x 10 mm card board strips.

2. One µl of 25 µg per ml of rLip L32 diluted in carbonate bicarbonate buffer (pH 9.6) was applied, air dried and fixed by incubating at 80°C for 30 minutes.

3. The unsaturated sites in the dipsticks were blocked by incubating for 1 hour in 5% dried milk powder in PBST at 37°C incubator.

4. The dipsticks were washed three times with an excess of PBST.

5. The dipsticks were incubated in serum samples diluted 1:100 in PBST at 37°C for 1 hour.

6. The positive and negative control were included along with each test.

7. The unbound proteins were removed by rinsing the dipsticks in PBST three times.

8. The dipsticks were incubated in diluted peroxidase conjugate at 37°C for 1 hour.

9. The dipsticks were washed three times with excess amount of PBST.

10. The dipsticks were dipped in substrate solution and incubated at room temperature for 5-10 minutes and observed the change in colour of the substrate solution.

Intensity of the colour was taken as indicator of the concentration of antibody.

3.2.9.3 Latex agglutination test (LAT)

Antigen for LAT

Purified recombinant Lip L32 antigen was diluted 50 μg per ml in carbonate bicarbonate buffer (pH 9.6).

Sensitization of Latex beads

Latex beads, 0.88 μ (Sigma, USA) were sensitized with recombinant Lip L32 antigen as per the methods of Lengyel *et al.* (1993) and Ramadass *et al.* (1999) with some modifications.

Latex beads 10% suspension, 50 μl were washed in carbonate bicarbonate buffer (pH 9.6) twice by centrifugation at 8,000 rpm for 3 min each time. The final latex beads were made 2 percent with carbonate bi-carbonate buffer and mixed with an equal volume of recombinant Lip L32 antigen diluted in carbonate bicarbonate buffer. The mixture was incubated at 37°C for 6 hours with constant shaking at 1500 rpm. The sensitized beads were centrifuged (8000 rpm) for 3 min. and the pellet resuspended as a 2 percent suspension in phosphate buffered saline (PBS) containing 5 mg/ml of bovine serum albumin (BSA). The latex beads were left at 37°C water bath overnight. The beads were centrifuged as before and the pellet was resuspended in PBS containing 0.5 mg/ml of BSA and 0.1 percent sodium azide as a 0.5 per cent suspension. Sensitized latex beads were stored at 4°C until use.

Conduct of Latex Agglutination test

Latex Agglutination test was preformed on 12 well glass slide. Twenty µl of sensitized latex beads solution and equal volume of serum sample were mixed thoroughly and slide was gently rocked for 2-5 min. PBS and proven positive serum were taken an negative and positive controls, respectively.

3.2.10 Microscopic agglutination test (MAT)

Microscopic agglutination test was conducted as per the method of Cole *et al.* (1973) with some modifications. In initial screening phase, test serum samples were diluted 1:50 and an equal amount of culture were mixed and plates were incubated for 2 hr at 37°C. Samples showing more than 50 per cent agglutination were considered positive.

The positive serum samples were then serially diluted and were tested using culture from serovars for which the particular sample was showing positive reaction. This second phase gave the titre of the antibodies against *Leptospira* in the serum.

3.2.11 Statistical analysis

The sensitivity, specificity and accuracy of each test like single serum dilution ELISA, Dipstick ELISA and LAT were compared with the standard test, MAT and calculated using the following formulae:

Sensitivity = a / (a+c)
Specificity = d / (b+d)
Accuracy = a + d / (a+b+c+d)

The results obtained from the tests were analysed for the percentage of agreement with MAT with the use of the Kappa statistics. The kappa statistics is a decimal measure of agreement between two tests especially in the absence of a standard and is defined as kappa or K.

K = (a + d - P) / 1 - P, where P = (a + b) (a + c) + (c + d) (b + d) and P is the probability, a is the number of samples positive by both ie test to be compared and MAT, b is the number of samples positive by MAT whereas negative by the test to be compared, c is the number of samples negative by MAT and positive by the test to be compared and d is the number of samples negative by both MAT and the test compared with MAT.

Guidelines for interpretation of K values are as follows:

k value < 0.1 indicates poor agreement
k value between 0.1 and 0.2 indicates slight agreement
k value between 0.21 and 0.4 indicates fair agreement
k value between 0.41 and 0.60 indicates moderate agreement
k value between 0.61 and 0.8 indicates substantial agreement
k value > 0.81 indicates perfect agreement

3.2.12 Indirect competitive assay for antigen detection using recombinant Lip L32 antigen

3.2.12.1 Indirect competitive ELISA

Five to seven days old leptospiral culture was washed with PBS, recombinant Lip L32 antigen and clinical sample were used as competitor antigens to compete with the recombinant Lip L32 antigen, attached to the solid phase. The method of Crowther, (1995) was followed with minor modifications.

Dilution steps for antigen : Recombinant Lip L32 (50 ng per well) was added to the first column and serially diluted to other columns, ending with column 11, so that each row had the serial two fold dilution of antigen. The plate was left overnight at 4°C for coating.

Dilution steps for antibody: The positive serum sample was initially diluted 1:100, and 100 μl was added into the first row (A1- A12) of the plate. The antibody from the first row was serially diluted to other rows.

The anti-human IgG HRP conjugate was added to all wells and indirect ELISA was carried out. From the data, the optimum dilution of antigen to be coated and antibody was determined and competitive assay was performed.

1. The plate was coated with 50 ng of recombinant Lip L32 antigen and left overnight at 4°C.

2. The plate was washed three times with PBST and blocked with 100 μl of 2% bovine serum albumin.

3. At the same time, 50 μl of the competitive antigen was incubated separately with 50 μl serum (1:200) for 1 hour at 37°C.

4. After blocking, the plates were washed and 100 μl of antigen and antibody mixture was added except for two wells and the plate incubated at 37°C for 1 hour. The positive and negative controls were included in this assay.

5. The conjugate and substrate was added as described in Indirect ELISA and the reading was taken at 405 nm.

Processing of Data: The positive control should get most colour, representing 0% competition. The wells in which no antibody and antigen (competitor) was added should represent 100% competition level in which there is a total inhibition of the binding of antibody.

3.2.12.2 Indirect competitive Dipstick ELISA

One µl of 25 µg/ml of recombinant Lip L32 antigen was applied on the strips and incubated at 80°C for 30 minutes. The unbound sites were blocked with 5% dried milk powder and incubated at 37°C for 1 hour. At the same time, 1 ml of leptospiral culture and positive clinical samples were incubated with the antibody (1:100) for 1 hour at 37°C. The strips were washed with PBST and incubated with the competitor antigen and antibody mixture for 1 hour at 37°C. The positive and negative control as included in Dipstick ELISA described in section 3.2.9.2 were also included in each test. The anti-human IgG HRP conjugate and substrate was added and the colour development was observed.

Results

4. RESULTS

4.1 Culturing Of Leptospires

The stock cultures of the leptospiral species viz. *pomona, autumnalis, hebdomadis, icterohaemorrhagiae, javanica, grippotyphosa, hardjo, canicola, patoc* and *andamana* were routinely subcultured at 8 weeks interval in semisolid EMJH media and maintained at room temperature in a dark place throughout the study. The liquid EMJH media was used for culturing the leptospiral organisms for DNA extraction, followed by PCR analysis. The concentration of the organisms were approximately 5 x 10^8 cells/ml in a 5-7 days-old culture under dark - field microscope.

Purification of isolates was carried out using dilution method. The dilutions of 5-7 days old isolate cultures in liquid medium were made from 10^{-1} to 10^{-7} using sterile PBS. Fifty μl volume from 10^{-4} to 10^{-7} dilutions were inoculated into semisolid EMJH medium and incubated. Growth of the organisms seen in the higher dilution as a clear subsurface ring known as Dinger's ring was observed in semisolid medium.

4.2 DNA Extraction

The high salt method was slightly modified for extraction of leptospiral DNA. The entire procedure took only 2-3 hours for the extraction of the DNA from leptospiral cultures.

The purity and concentration of DNA samples were checked by UV spectrophotometer analysis at 260 nm and 280 nm wavelengths and agarose gel electrophoresis. In the agarose gel (0.8% w/v) electrophoresis, the DNA samples were visualized as distinct band. But RNA bands were also observed. To reduce the quantity of RNA, RNase (10-20 μg/ml) was added. In the UV spectrophotometer analysis, A_{260}/A_{280} ratio was found to be between 1.7 and 1.8 for the DNA samples.

The high salt method was found to be rapid, and followed to extract the leptospiral DNA samples for PCR analysis. All the DNA samples were diluted to the concentration of approximately 100 ng of DNA/μl volume.

4.3 Polymerase Chain Reaction for the Amplification of Partial Lip L32 Gene

The forward and reverse primers specific for the pathogenic serovars of *Leptospira* were designed in such a way that following amplification, the amplified product shall have restriction enzyme sites viz., *Xba*I and *Kpn*I at the 5'ends of the PCR product respectively. The primers were designed from the available sequence of *Leptospira kirschneri* serovar. Ten μl aliquot of the amplified product was electrophoresed in 1.2% agarose gel along with 100 bp DNA ladder. The product of 665 bp was obtained (Plate 1). The primers could amplify the pathogenic serovars of Leptospira like *pomona, autumnalis, javanica, icterohaemmorhagiae* but non-pathogenic serovars like *patoc* and *andamana* could not be amplified (Plate 1). The amplified product of 665 bp from the serovar *pomona* was purified and used for cloning in the cloning and expression vectors.

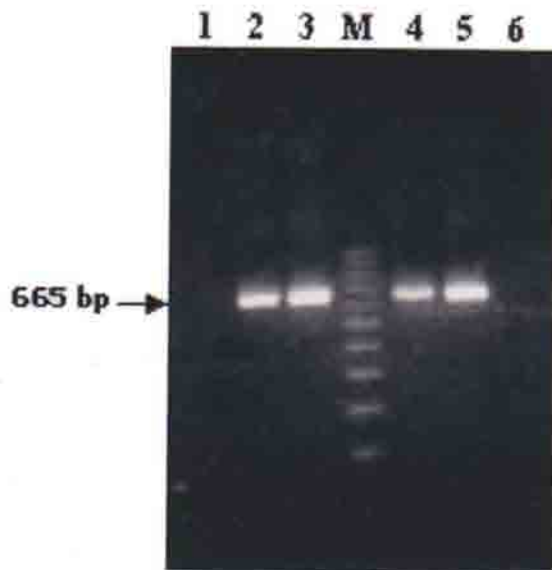


Plate 1 : Presence of the Lip L32 gene among the various leptospiral serovars, electrophoresed in 1.2% agarose gel

Lane 1 : *Leptospira andamana*
Lane 2 : *Leptospira pomona*
Lane 3 : *Leptospira icterohaemorrhagiae*
M : 100 bp DNA ladder
Lane 4 : *Leptospira hardjo*
Lane 5 : *Leptospira javanica*
Lane 6 : *Leptospira patoc*

4.4 Cloning of the Lip L32 gene into pBluescript II KS (+) Vector

The Lip L32 gene was cloned into pBluescript II KS (+) phagemid vector by blunt end ligation. pBluescript II KS (+) is a cloning vector, flanked by T3 and T7 RNA promoters. The uncut plasmid migrated as 3 bands in 0.8% (w/v) agarose gel due to the occurrence of 3 forms of plasmids.

4.4.1 Linearization of pBluescript II KS (+) phagemid vector

The cloning vector was linearized with *SmaI* restriction enzyme to create blunt ends and it migrated at 3 Kb level in 0.8% (w/v) agarose gel. The linearized plasmid was purified with DNA extraction kit and used for cloning.

4.4.2 Ligation and transformation

The T4 DNA ligase was used to ligate the blunt ends of pBluescript II KS (+) vector and filled up the amplicon, Lip L32 gene. Two ligation reactions were set; one reaction with the PCR product as insert and the other without the PCR product (Ligation control). A vector: insert (V:I) molar ratio of 1:3 was used for ligation. 1 to 3 μl of the reaction mixture was transformed into DH5α cells. The transformants were spread onto LB ampicillin X-gal/IPTG plates for recombinant selection. White colonies were supposed to harbour the recombinant plasmid while blue colony harbours the recircularized plasmids. The white colonies were then streaked onto ampicillin plates.

4.4.3 Colony PCR

The pBluescript T3 primer as forward and reverse gene specific primer were used to confirm the orientation of the insert. Total 2 out of 20 colonies yielded an expected amplified product of 665 bp in 1.2% agarose gel. Out of the 2 colonies, 1 was in right orientation with a size of 759 bp as amplified by T3 and reverse gene specific primer (Plate 2). The clone designated as pKSP4 was used for further characterization.

4.4.4 Restriction enzyme digestion of the recombinant plasmid

The recombinant plasmids that were around 3.6 kb in size were subjected to digestion with *Eco*RI/*Xba*I and *Kpn*I to confirm the presence of the insert and also to check the orientation of the insert.

Digestion of the recombinant plasmid with *Eco*RI linearized the plasmid. Digestion with *Eco*RI/*Kpn*I, released the insert of approximately 680 bp and the vector of 2.9 kb when compared with the λ DNA - *Hind*III and ϕ x 174 DNA *Hae*III double digest marker (Plate 3). This indicated that the Lip L32 gene has been cloned upstream in the cloning vector. However, final confirmation was made by sequencing the recombinant plasmid.

Digestion of the recombinant plasmid with *Xba*I and *Kpn*I resulted in the plasmid of around 2.9 kb and released the insert of 665 bp as the sites for these restriction enzymes were designed at 5' end of the forward and reverse primer specific for Lip L32 gene. The insert of 665 bp was again subcloned into the expression vector.

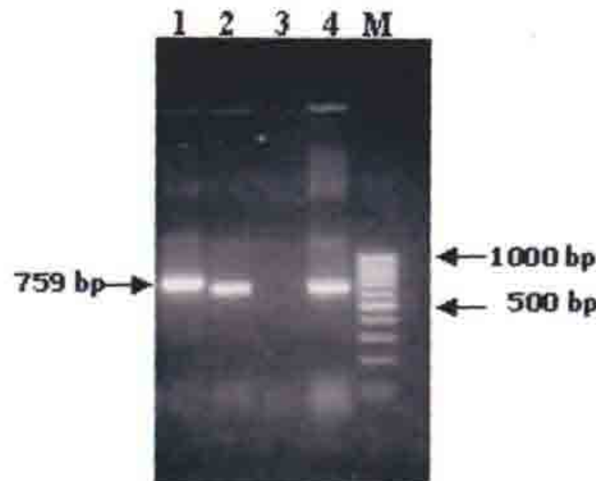


Plate 2 : Colony PCR for identifying the Lip L32 gene in pBluescript II KS (+) vector, electrophoresed in 1.2% agarose gel

Lane 1 : pKSP4 clone amplified with T3 forward and reverse gene specific primers (759 bp)
Lane 2 : pKSP4 clone amplified with forward and reverse gene specific primers (665 bp)
Lane 3 : Recombinant clone 2 amplified with T3 forward and reverse gene specific primers (incorrect orientation)
Lane 4 : Recombinant clone 2 amplified with forward and reverse gene specific primers (665 bp)
M : 100 bp ladder

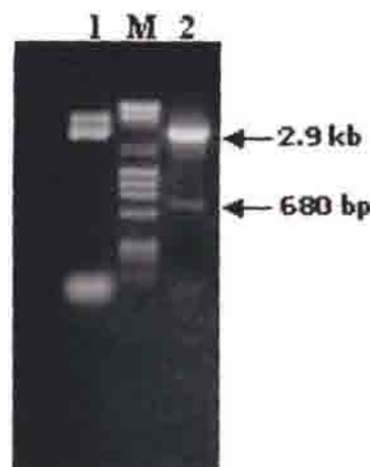


Plate 3 : Restriction enzyme digestion to confirm the presence of insert in the recombinant clone (pKSP4) electrophoresed in 1.2% agarose gel

Lane 1 : pKSP4 uncut recombinant plasmid
M : λDNA *Hind*III / ϕX 174 DNA *Hae*III Marker
Lane 2 : pKSP4 recombinant plasmid digested with *Eco*RI and *Kpn*I

4.5 Sequencing

4.5.1 Preparation of the recombinant plasmid for sequencing

The recombinant plasmid pKSP4 was prepared by inoculating the colony into LB ampicillin broth. Plasmid was eluted in 20 μl of nuclease free water. Then it was purified with the DNA extraction kit and run on 0.8% agarose gel to check the size and purity. The concentration of the recombinant plasmid was adjusted to 100 ng per μl for sequencing.

4.5.2 Sequencing of the recombinant plasmid

The recombinant plasmid pKSP4 was sequenced with the pBluescript T3 sequencing primer and reverse gene specific primer and sequences until 450 bp could be read. Following, sequencing with pBluescript T7 sequencing primer and forward gene specific primers, sequences until 400 bp could be read. Using the GeneTool software, the T7 sequencing primer - generated sequence was reverse complemented and aligned with the T3 sequencing primer sequence to generate the complete nucleotide sequence. The complete sequence was stored as a text file in FASTA format and used for further analysis. The nucleotide and amino acid sequence has been deposited in the GenBank under the accession number AY223718 (Fig.1).

4.6 Sequence Analysis

4.6.1 Phylogenetic analysis of the obtained Lip L32 sequence with software Clustal X and DNASTAR

The nucleotide sequence of Lip L32 of *Leptospira interrogans* serovar *pomona* strain Pomona (L.pomona) was stored along with the nucleotide sequence of other pathogenic *Leptospira* serovars in FASTA format. The sequences were then loaded onto the programme to obtain the alignment file and dendrogram file.

```
gct cta gat atc tcc gtt gca ctc ttt gca agc att acc gct tgt ggt gct ttc   54
ALA LEU ASP ILE SER VAL ALA LEU PHE ALA SER ILE THR ALA CYS GLY ALA PHE   18

ggt ggt ctg cca agc cta aaa agc tct ttt gtt ctg agc gag gac aca atc cca  108
GLY GLY LEU PRO SER LEU LYS SER SER PHE VAL LEU SER GLU ASP THR ILE PRO   36

ggg aca aac gaa acc gta aaa acg tta ctt ccc tac gga tct gtg atc aac tat  162
GLY THR ASN GLU THR VAL LYS THR LEU LEU PRO TYR GLY SER VAL ILE ASN TYR   54

tac gga tac gta aag cca gga caa gcg ccg gac ggt tta gtc gat gga aac aaa  216
TYR GLY TYR VAL LYS PRO GLY GLN ALA PRO ASP GLY LEU VAL ASP GLY ASN LYS   72

aaa gca tac tat ctc tat gtt tgg att cct gcc gta atc gct gaa ata gga gtt  270
LYS ALA TYR TYR LYS TYR VAL TRP ILE PRO ALA VAL ILE ALA GLU ILE GLY VAL   90

cgt atg att tcc cca aca ggc gaa atc ggt gaa cca ggc gac gga gac tta gta  324
ARG MET ILE SER PRO THR GLY GLU ILE GLY GLU PRO GLY ASP GLY ASP LEU VAL  108

agc gac gct ttc aaa gcg gct acc cca gaa gaa aaa tca atg cca cat tgg ttt  378
SER ASP ALA PHE LYS ALA ALA THR PRO GLU GLU LYS SER MET PRO HIS TRP PHE  126

gat act tgg atc cgt gta gaa aga atg tcg gcg att atg cct gac caa atc gtc  432
ASP THR TRP ILE ARG VAL GLU ARG MET SER ALA ILE MET PRO ASP GLN ILE VAL  144

aaa gct gcg aaa gca aaa cca gtt caa aaa ttg gac gat gat gat gat ggt gac  486
LYS ALA ALA LYS ALA LYS PRO VAL GLN LYS LEU ASP ASP ASP ASP ASP GLY ASP  162

gat act tat aaa gaa gag aga cac aac aag tac aac tct ctt act aga atc aag  540
ASP THR TYR LYS GLU GLU ARG HIS ASN LYS TYR ASN SER LEU THR ARG ILE LYS  180

atc cct aat cct cca aaa tct ttt gac gat ctg aaa aac atc gac act aaa aaa  594
ILE PRO ASN PRO PRO LYS SER PHE ASP ASP LEU LYS ASN ILE ASP THR LYS LYS  198

ctt tta gta aga ggt ctg tac aga att cca ttc act acc tac aaa cca ggt gaa  648
LEU LEU VAL ARG GLY LEU TYR ARG ILE PRO PHE THR THR TYR LYS PRO GLY GLU  216

gtg aaa gga ggt acc                                                       663
VAL LYS GLY GLY THR                                                       221
```

Fig. 1. Lip L32 gene sequence of *Leptospira pomona* aligned with the deduced amino acid sequence

(The carboxy-terminal ILE-THR-ALA-CYS signal peptidase II cleavage site is indicated by green colour)

Comparison of the cloned Lip L32 DNA sequence with other pathogenic leptospiral strains, revealed a high degree of sequence conservation with an average DNA sequence identity of 97.42% (range, 92.5 to 99.2%) (Fig.2). The percentage similarity and nucleotide divergence generated using the DNASTAR software is shown in Table 1.

The Neighbour - Joining (NJ) tree file produced by Clustal V method from the Meg Align package produced three groups (Fig.3). The group I consisted of *L.interrogans* and *L.kirschneri* species, group II was represented by *L.borgpetersenii* serovar *hardjo* strain 203 (AF 181554) and group III included *L.santarosai* serovar *tropica* strain CA 299 (AF 181555). The group I consisted of serovar *grippotyphosa* strain RM52 (AF 121192) of *L.kirschneri* species showed relatedness with *L.interrogans* species namely *pomona (AF 181553), australis (AB 094437), canicola (AB 094434), icterohaemmorhagiae (AY 423075)* and *autumnalis (AB 094435)* serovars. The Pomona and RZ 11 strains of serovar *pomona* showed no difference. The Hond Utrecht IV strain of serovar *canicola* and Akiyami C strain of serovar *australis* again showed no difference; the Akiyami A strain of serovar *autumnalis* and RGA strain of serovar *icterrohaemorrhagiae* showed similarity.

4.6.2 Amino acid sequence alignment and predicted characteristics of the cloned Lip L32 gene product

The Lip L32 amino acid sequence of the serovar *pomona* (Lpomona) obtained by conceptual translation was aligned with the other predicted sequences of *Leptospira* species (Fig.4). In the cloned Lip L32, the

Table 1. Nucleotide similarity and divergence of the sequences of serovars of *Leptospira*

	L. autum	L. borg.	L. canicola	L. ictero	L.kirsch RM52	L.santaros	L. australis	L.pomona RZ11	L.pomona	
L. autum	***	95.5	99.8	99.1	98.7	93.1	99.8	99.5	98.7	L. autum
L. borg.	4.5	***	95.6	95	95.8	94.2	95.6	95.6	94.8	L. borg.
L. canicola	0.2	4.4	***	98.9	98.9	93.3	100	99.7	98.9	L. canicola
L. ictero	0.6	4.9	0.8	***	98.3	92.6	98.9	99.1	98.3	L. ictero
L.kirsch RM52	1.3	4.2	1.1	1.6	***	92.8	98.9	98.9	98.1	L.kirsch RM52
L.santaros	6.9	5.9	6.8	7.3	7.3	***	93.3	93.3	92.5	L.santaros
L. australis	0.2	4.4	0	0.8	1.1	6.8	***	99.7	98.9	L. australis
L.pomona RZ11	0.5	4.4	0.3	0.8	1.1	6.8	0.3	***	99.2	L.pomona RZ11
L.pomona	1.1	5	0.9	1.4	1.7	7.5	0.9	0.6	***	L.pomona
	L. autum	L. borg.	L. canicola	L. ictero	L.kirsch RM52	L.santaros	L. australis	L.pomona RZ11	L.pomona	

Percent similarity in upper triangle

Percent divergence in lower triangle

```
L.pomona            GCACTCTTT GCAAGCATT ACCGCTTGT GGTGCTTTC GGTGGTCTG CCAAGCCTA AAAAGCTCT TTTGTTCTG AGCGAGGAC ACAATCCCA GGGACAAAC GAAACCGTA 108
L.pomona(RZ11)      ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.australis         ......... ....T.... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.canicola          ......... ....T.... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.ictero            ......... ......C.C ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.kirschneri(RM52)  ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.santarosai        ......... ......... ......... ......... ......... ........T ......... ......... ......AG. ...G.T..G ..A...... ...C..A..
L.borgpetersenii    ......... ......... ......... .....G... ......T.. ........T ......... ......... ......AG. ...G.T... ..A...... .........
L.autumnalis        ......... ....T.... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........

L.pomona            AAAACGTTA CTTCCCTAC GGATCTGTG ATCAACTAT TACGGATAC GTAAAGCCA GGACAAGCG CCGGACGGT TTAGTCGAT GGAAACAAA AAAGCATAC TATCTCTAT 216
L.pomona(RZ11)      ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.australis         ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.canicola          ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.ictero            ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.kirschneri(RM52)  ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.santarosai        ........C ........T ......... ......... ......... A........ ......... .....T..C ........C ......... ......... .........
L.borgpetersenii    ........C ........T ......... ......... ........T ......... ......... ......... ........C ......... ......... .........
L.autumnalis        ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........

L.pomona            GTTTGGATT CCTGCCGTA ATCGCTGAA ATAGGAGTT CGTATGATT TCCCCAACA GGCGAAATC GGTGAACCA GGCGACGGA GACTTAGTA AGCGACGCT TTCAAAGCG 324
L.pomona(RZ11)      ......... ......... ......... ..G...... ......... ......... ......... ......... ......... ......... ......... .........
L.australis         ......... ......... ......... ..G...... ......... ......... ......... ......... ......... ......... ......... .........
L.canicola          ......... ......... ......... ..G...... ......... ......... ......... ......... ......... ......... ......... .........
L.ictero            ......... ......... ......... ..G..T... ......... ......... ......... .....G... ......... ......... ......... .........
L.kirschneri(RM52)  ......... ......... ......... ..G...... ......... ......... ......... ......... .....T... ......... ......... .........
L.santarosai        ......... ..A..T... ......... ..G...... ......... ......... ......... ......... ......... ..T...... ..T..T..G .........
L.borgpetersenii    ........C ..A..T... ......... ..G...... ......... ......... ......... ..G...... ......... ......... ......... .........
L.autumnalis        ......... ......... ......... ..G...... ......... ......... ......... .....G... ......... ......... ......... .........

L.pomona            GCTACCCCA GAAGAAAAA TCAATGCCA CATTGGTTT GATACTTGG ATCCGTGTA GAAAGAATG TCGGCGATT ATGCCTGAC CAAATCGTC AAAGCTGCG AAAGCAAAA 432
L.pomona(RZ11)      ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.australis         ......... ......... ......... ......... ......... ......... ......... ......... ......... .......C. ......... .........
L.canicola          ......... ......... ......... ......... ......... ......... ......... ......... ......... .......C. ......... .........
L.ictero            ......... ......... ......... ......... ......... ......... ......... ......... ......... .......C. ......... .........
L.kirschneri(RM52)  ......... ......... ......... ......... ......... ......... ......... ......... ......... .......C. ......... .........
L.santarosai        ..A...... ......... ......... ......... ......... ...GC..CT ......... .....A... .....C... .......CT .....A... ....G....
L.borgpetersenii    ..A.....G ......... ......... ......... .....C... ......... ......... ......... ......... .......C. ......... .........
L.autumnalis        ......... ......... ......... ......... ......... ......... ......... ......... ......... .......C. ......... .........

L.pomona            CCAGTTCAA AAATTGGAC GATGATGAT GATGGTGAC GATACTTAT AAAGAAGAG AGACACAAC AAGTACAAC TCTCTTACT AGAATCAAG ATCCCTAAT CCTCCAAAA 540
L.pomona(RZ11)      ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.australis         ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.canicola          ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.ictero            ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........
L.kirschneri(RM52)  ..C...... ......... ......... ......... ......... ......... ........T ......... ......... ......... ......... .........
L.santarosai        ......... .......G. T....C... ........T ......... ..G...... ......... ..A...... .......T. ......... ......... .........
L.borgpetersenii    ..C...... ......A.. ......... ........T ......... ......... ........T ..A...... ......... ......... .....A... .........
L.autumnalis        ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... ......... .........

L.pomona            TCTTTTGAC GATCTGAAA AACATCGAC ACTAAAAAA CTTTTAGTA AGAGGTCTG TACAGAATT CCATTCACT ACCTACAAA CCAGGTGAA GTGAAAGGA     639
L.pomona(RZ11)      ......... ......... ......... ......... ......... ........T ......... T.T...... ......... ......... .........
L.australis         ......... ......... ......... ......... ......... ........T ......... T.T...... ......... ......... .........
L.canicola          ......... ......... ......... ......... ......... ........T ......... T.T...... ......... ......... .........
L.ictero            ......... ......... ......... ......... ......... ........T ......... T.T...... ......... ......... .........
L.kirschneri(RM52)  ......... ..C...... ........T ......... ......... ........T ......... T.T...C.. ......... ......... .........
L.santarosai        ......... ......... ........T ......... ......... ........T ......... T.T...... ......... ..G...... .........
L.borgpetersenii    ......... ......... ........T ......... ......... ........T ......... T.T...... ......... ......... .........
L.autumnalis        ......... ......... ......... ......... ......... ........T ......... T.T...... ......... ......... .........
```

Fig. 2. Nucleotide sequence of *Leptospira interrogans* serovar *pomona* in comparison with the other serovars of *Leptospira*

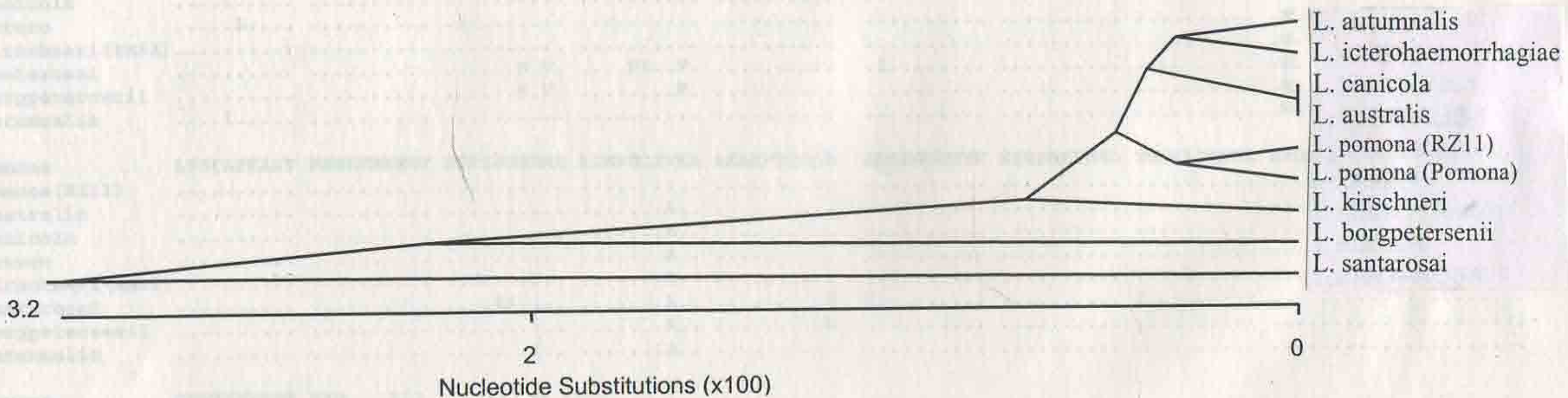


Fig.3 Phylogenetic tree based on the nucleotide sequence alignment of the Lip L32 gene of different serovars of *Leptospira*

```
L.pomona            ALFASITACG AFGGLPSLKS SFVLSEDTIP GTNETVKTLL PYGSVINYYG YVKPGQAPDG LVDGNKKAYY LYVWIPAVIA EIGVRMISPT GEIGEPGDGD 100
L.pomona(RZ11)      .......... .......... .......... .......... .......... .......... .......... .......... .M........ ..........
L.australis         ....I..... .......... .......... .......... .......... .......... .......... .......... .M........ ..........
L.canicola          ....I..... .......... .......... .......... .......... .......... .......... .......... .M........ ..........
L.ictero            .....L.... .......... .......... .......... .......... .......... .......... .......... .M........ ..........
L.kirschneri(RM52)  .......... .......... .......... .......... .......... .......... .......... .......... .M........ ..........
L.santarosai        .......... .......... ......S.V. ....PI..F. .......... .I........ .......... .......... .M........ ..........
L.borgpetersenii    .......... .......... ......S.V. ........F. .......... .......... .......... .......... .M........ ..........
L.autumnalis        ....I..... .......... .......... .......... .......... .......... .......... .......... .M........ ..........

L.pomona            LVSDAFKAAT PEEKSMPHWF DTWIRVERMS AIMPDQIVKA AKAKPVQKLD DDDDGDDTYK EERHNKYNSL TRIKIPNPPK SFDDLKNIDT KKLLVRGLYR 200
L.pomona(RZ11)      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
L.australis         .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
L.canicola          .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
L.ictero            .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
L.kirschneri(RM52)  .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
L.santarosai        .......... .......... ....AA.... .......A.. .........G Y......... .......... I......... .......... ..........
L.borgpetersenii    .......... .......... .......... .......A.. .........N .......... .......... .......... .......... ..........
L.autumnalis        .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........

L.pomona            IPFTTYKPGE VKG    213
L.pomona(RZ11)      .S........ ...
L.australis         .S........ ...
L.canicola          .S........ ...
L.ictero            .S........ ...
L.kirschneri(RM52)  .S.P...... ...
L.santarosai        .S........ ...
L.borgpetersenii    .S........ ...
L.autumnalis        .S........ ...
```

Fig. 4. Amino acid sequence of *Leptospira interrogans* serovar *pomona* in comparison with the other serovars of *Leptospira*

amino acid isoleucine has been substituted with serine at # 5 in the serovars *pomona* (RZ11), *santarosai, kirschneri* (RM52), *icterohaemorrhagiae* (RGA) and *borgpetersenii*. At # 82, methionine has been substituted with isoleucine in L.pomona whereas the amino acid alanine has been substituted with valine at # 138 and at # 202, serine has been substituted with proline. Otherwise, comparison of the deduced amino acid sequences of the Lip L32 variants revealed an average amino acid sequence identity of 98.06% (range, 96.71 to 99.06%). Most of the sequence polymorphisms detected (39 of 56) were silent.

As expected for a lipoprotein, the deduced amino acid sequence begins with the carboxy - terminal I-T-A-C signal peptidase II cleavage site. Beginning at # 150 there is an unusual cluster of seven aspartate residues in a span of eight amino acids.

4.6.3 Open reading frame (ORF) and protein sequence determination

The GeneTool software - based analysis revealed only one open reading frame, which was able to code for a polypeptide of 221 amino acids. Other frames could not encode any longer polypeptide

4.7 *In vitro* Expression of Lip L32 Protein

The purified Lip L32 gene (665 bp) subcloned from pBluscript II KS (+) and ligated to pPro Ex HT 'a' expression vector which is 4779 bp in size.

4.7.1 Recombinant selection

The M13 reverse sequencing primer and reverse gene - specific primer were used to confirm the orientation of the insert. Two out of 12 colonies yielded an expected amplified product of 830 bp in 1.2% agarose gel (Plate 4). The two colonies were also checked with the forward and reverse gene specific primer and an expected size of 665 bp amplicon was observed in 1.2% agarose gel (Plate 4). The clone designated as pPro10 was used for expression.

The recombinant plasmid pPro10 that was around 5.4 kb in size was subjected to digestion with *Eco*RI and *Hind*III to confirm the presence of the insert of size 720 bp (Plate 5).

4.7.2 Induction of Lip L32 recombinant protein

The recombinant plasmid, pPro10 was transformed into *Escherichia coli* DH5α cells. Expression from the trc promoter was induced by 1 mM IPTG. Protein kinetic studies in 12% SDS - PAGE indicated that the maximal expression was between second and third hours interval post induction (Plate 6). A fusion protein (221 amino acids from Lip L32 and residues from the fusion tag) of size 32 kDa was expected.

4.8 Characterization and Purification of the Recombinant Lip L32 Protein

4.8.1 Western blot technique

To address the level and distribution of Lip L32 expression, immunoblot analysis was performed using hyperimmune serum from the mice immunized with the serovar *pomona* (Plate 7). The antiserum was reactive with

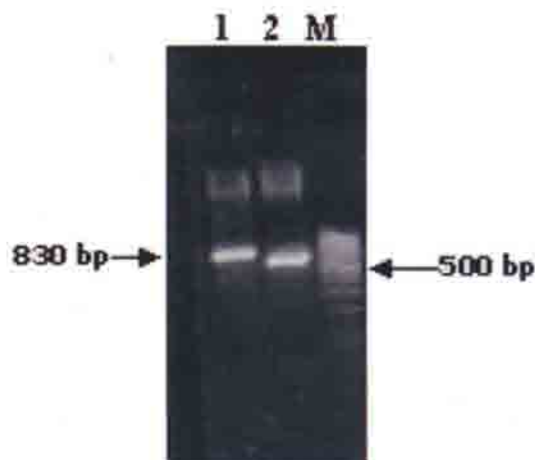


Plate 4 : Colony PCR for detecting the presence of Lip L32 gene in pPro EX HT 'a' vector electrophoresed in 1.2% agarose gel

Lane 1 : Recombinant clone (pPro10) amplified with M13 reverse sequencing primer and reverse gene specific primer
Lane 2 : Recombinant clone (pPro10) amplified with forward and reverse gene specific primers (665 bp)
M : 100 bp DNA ladder

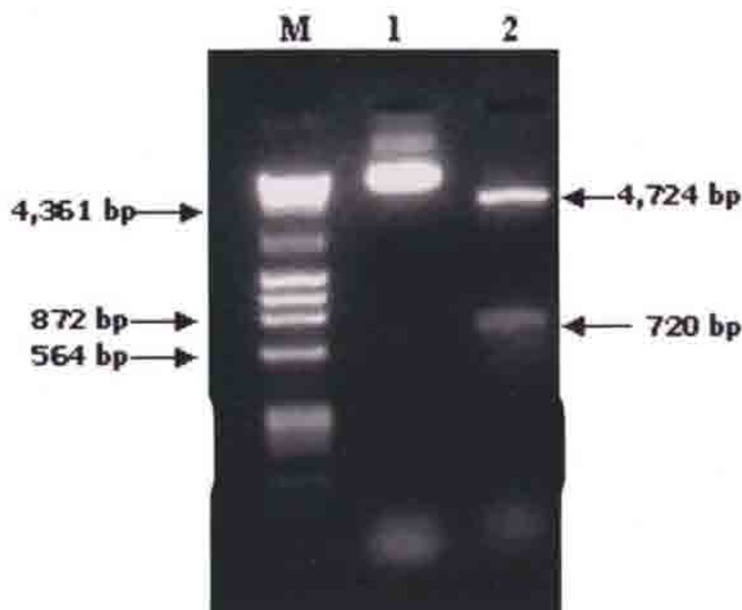


Plate 5 : Restriction enzyme digestion to confirm the presence of Lip L32 in the recombinant clone (pPro10) electrophoresed in 1.2% agarose gel

M : λ DNA *Hind*III /φX174 *Hae*III double digest Marker
Lane 1 : Recombinant plasmid (pPro10)
Lane 2 : Recombinant plasmid (pPro10) digested with *Eco*RI and *Hind*III

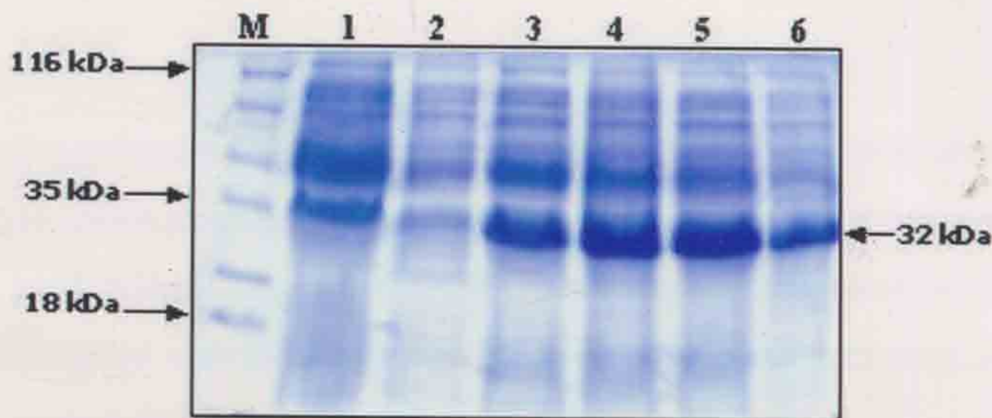


Plate 6 : Protein kinetics study of recombinant Lip L32 in 12% SDS - PAGE

M : Protein Molecular Weight Marker
Lane 1 : *E.coli* DH5α cells harbouring the plasmid pPro EX HT 'a', IPTG (+)
Lane 2 : *E.coli* DH5α cells harbouring the recombinant clone (pPro10), IPTG (–)
Lane 3 : *E.coli* DH5α cells harbouring the recombinant clone (pPro10), IPTG (1 hr post – induction)
Lane 4 : *E.coli* DH5α cells harbouring the recombinant clone (pPro10), IPTG (2 hr post – induction)
Lane 5 : *E.coli* DH5α cells harbouring the recombinant clone (pPro10), IPTG (3 hr post – induction)
Lane 6 : *E.coli* DH5α cells harbouring the recombinant clone (pPro10), IPTG (4 hr post – induction)

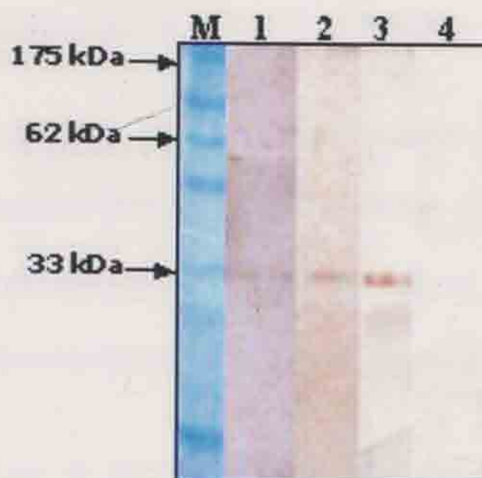


Plate 7 : Analysis of the recombinant Lip L32 protein by Western Blot technique

M : Prestained Protein Marker
Lane 1 : Immunoblotting with human serum sample
Lane 2 : Immunoblotting with dog serum sample
Lane 3 : Immunoblotting with hyperimmune serum raised against serovar *pomona*
Lane 4 : Immunoblotting with hyperimmune serum raised

a single band of molecular mass of approximately 32 kDa. The positive canine and human serum samples also reacted with the Lip L32 antigen and showed a single band of 32 kDa size. However, when the hyperimmune serum from the rabbit immunized with the non-pathogenic serovar *patoc* was performed, no protein band of 32 kDa was detected.

4.8.2 Purification of the recombinant Lip L32 protein

For outer membrane protein, 8 M urea was used to solubilize the culture pellet. The recombinant Lip L32 fusion protein was purified by affinity chromatography. The 6x Histidine fusion protein was bound to the immobilized nickel (Ni^{2+}) column and the recombinant fusion protein was eluted maximally with 500 mM imidazole as analyzed in 12% SDS-PAGE (Plate 8).

4.8.3 Concentration of the recombinant Lip L32 protein

The concentration of the recombinant protein was determined as 0.4 gm % with an absorbance of 0.011 at A555.

4.9 Detection of Leptospiral Antibodies in Clinical Samples by Recombinant Lip L32 Antigen

4.9.1 Enzyme - linked immunosorbent assay (ELISA)

4.9.1.1 Standardization of recombinant antigen ELISAs

Prior to use in ELISAs, the recombinant antigen was tested in immunoblots: pooled sera from leptospirosis cases (human and dog) had strong reactions to recombinant Lip L32 antigen. The performance of the recombinant

IgG ELISA at different antigen concentrations and serum dilution (1:100) is shown (Fig. 5). Maximum absorbance values were observed at a concentration of 50 ng/well, while absorbance decreased with increasing antigen concentration. The optimum concentration of the conjugate was determined in human and dog clinical samples as 1:4000 and 1:3000, respectively.

4.9.1.2 Serial dilution ELISA

Absorbance of serial dilutions of sera were measured and antibody titres were calculated by the substraction method as described. The end-point titres for thirty five dog and human sera were calculated by plotting the average of triplicate, corrected absorbance readings at serial dilutions until the PNT baseline was intersected (Figs.6 and 7).

4.9.1.3 Predicted ELISA antibody titres

A linear relationship existed between the log of the corrected absorbance of dog and human sera at a single working dilution 1:1000 and 1:250, respectively and the corresponding observed titres as determined by serial dilution. Regression analysis yielded a regression line with a correlation coefficient of 0.967 in dogs (Fig. 8) and 0.864 in humans (Fig. 9). The average absorbances at 1:1000 and 1:250 for dog and human sera, respectively were corrected by the substraction method and the predicted titres were determined using the regression line equation, $\log_{10}$ (S/P ratio) = intecept + slope x $\log_{10}$ (titre) and depicted in Figs. 8 and 9 for dog and human sera samples, respectively.

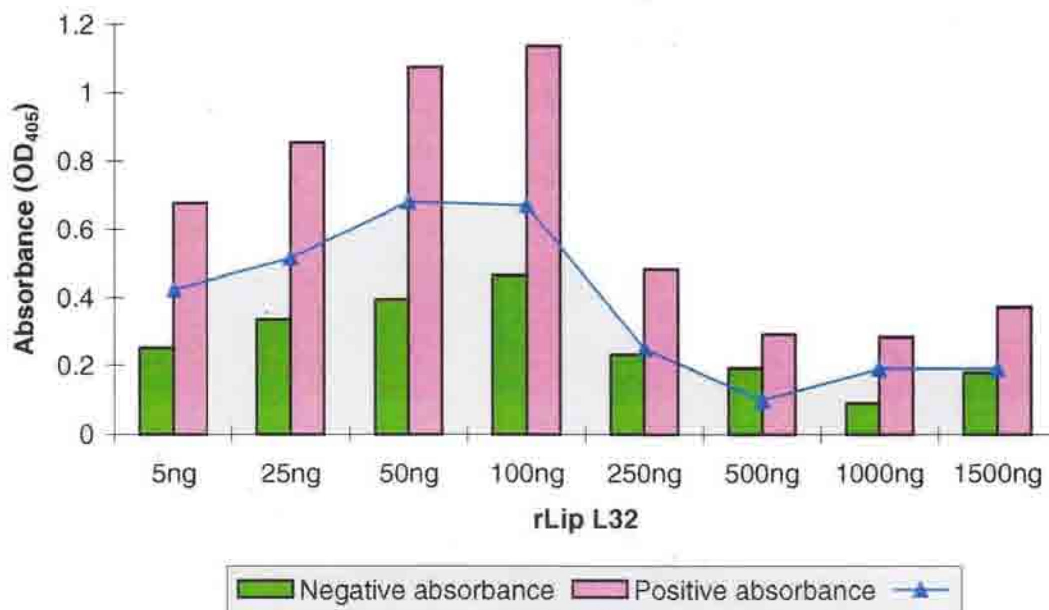


Fig. 5. Evaluation and standardization of the recombinant antigen concentration (5 ng, 25 ng, 50 ng, 100 ng, 250 ng, 500 ng, 1 μg, 1.5 μg) at a serum dilution of 1: 100

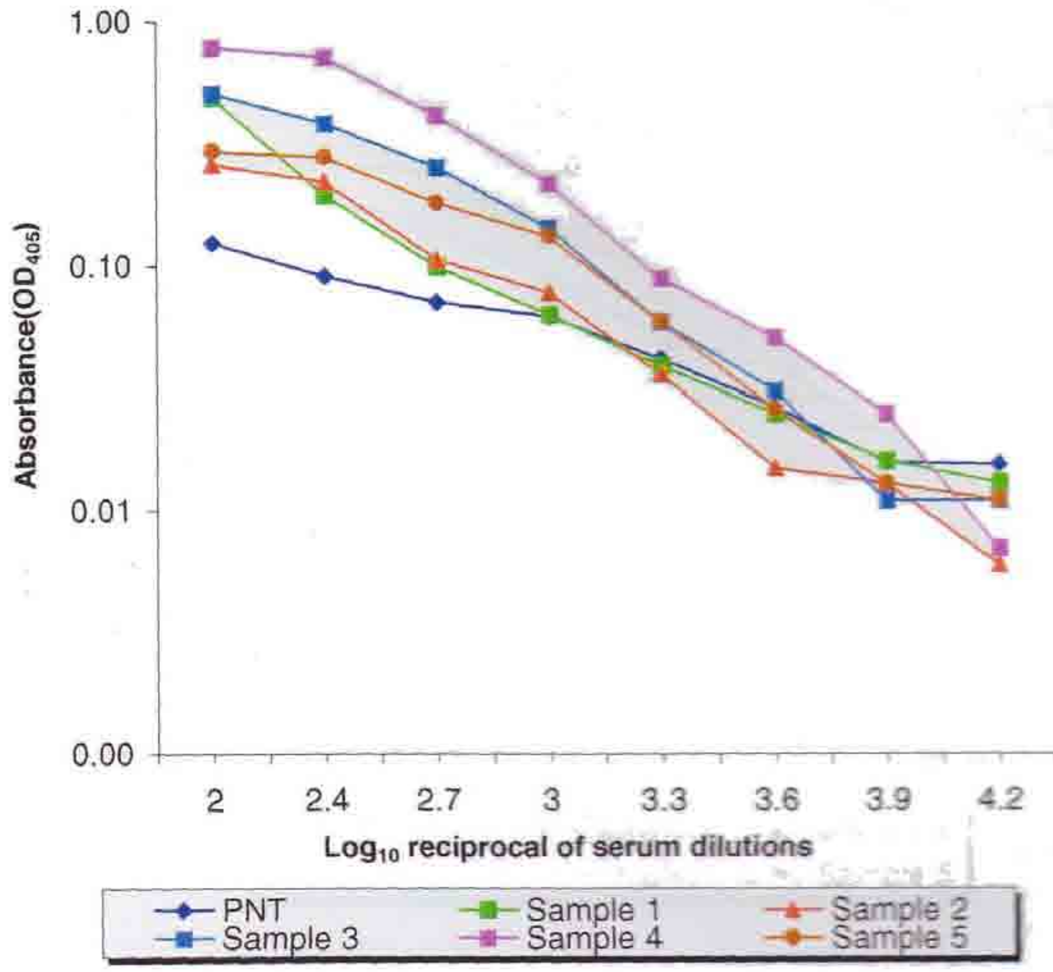


Fig. 6. Representation of the positive- negative threshold (PNT) baseline with different positive dog serum samples

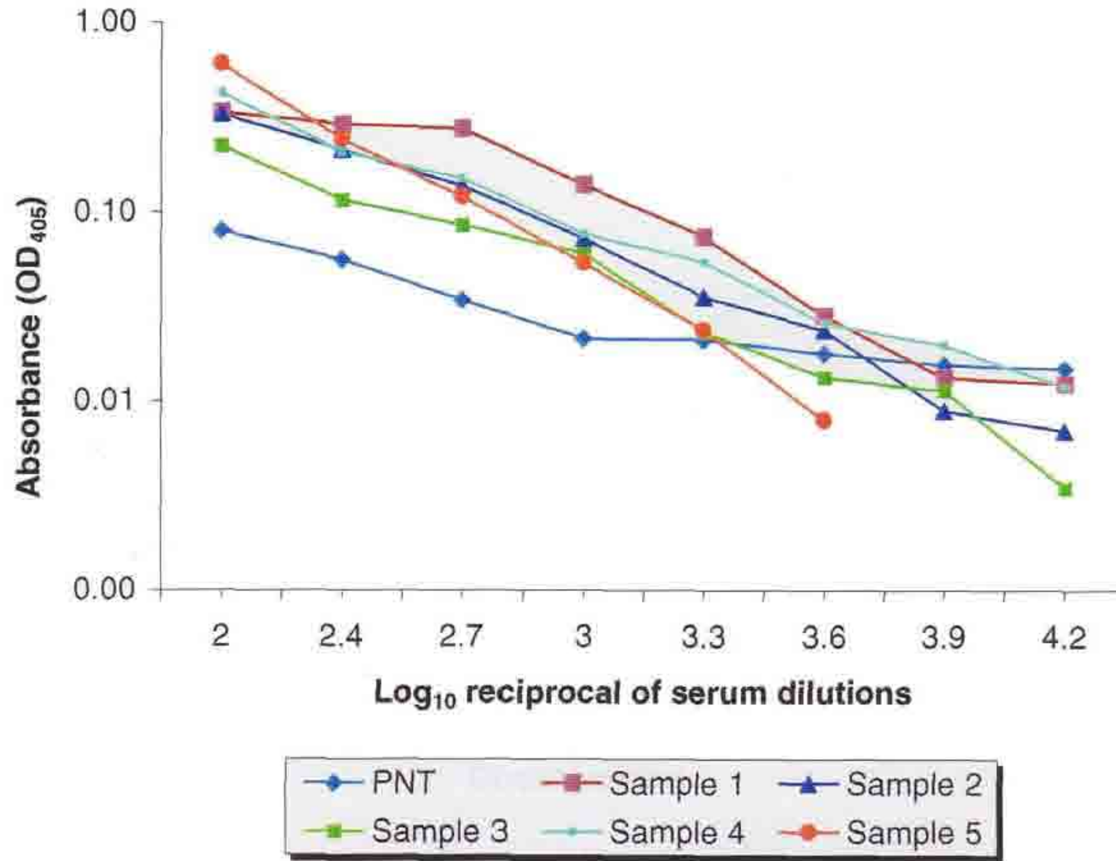


Fig. 7. Representation of the positive- negative threshold (PNT) baseline with different human positive serum samples

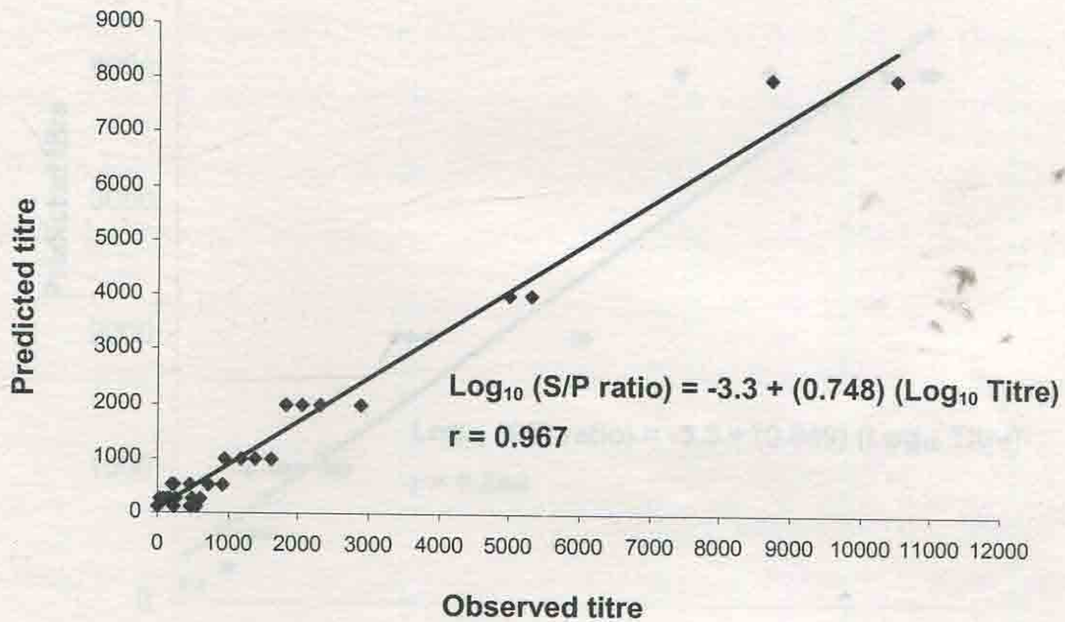


Fig. 8. Correlation between the observed antibody titres obtained from serial dilution ELISA and the predicted antibody titre obtained from single dilution ELISA at a 1: 1000 dilution

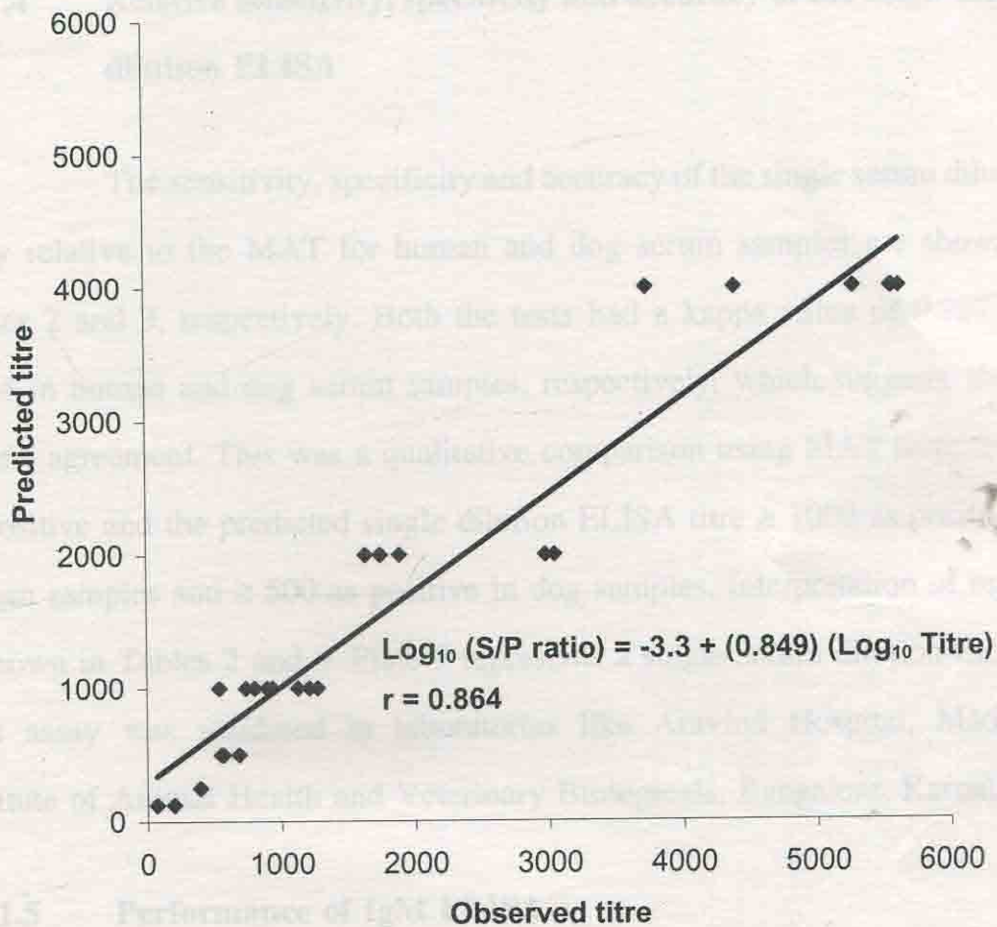


Fig. 9. Correlation between the observed antibody titres obtained from serial dilution ELISA and the predicted antibody titres obtained from single dilution ELISA at a 1: 250 dilution

4.9.1.4 Relative sensitivity, specificity and accuracy of the single serum dilution ELISA

The sensitivity, specificity and accuracy of the single serum dilution assay relative to the MAT for human and dog serum samples are shown in Tables 2 and 3, respectively. Both the tests had a kappa value of 0.987 and 0.984 in human and dog serum samples, respectively, which suggests almost perfect agreement. This was a qualitative comparison using MAT titres ≥ 100 as positive and the predicted single dilution ELISA titre ≥ 1000 as positive in human samples and ≥ 500 as positive in dog samples. Interpretation of results is shown in Tables 2 and 3. Plate 9 represents a single serum dilution ELISA. This assay was validated in laboratories like Aravind Hospital, Madurai, Institute of Animal Health and Veterinary Biologicals, Bangalore, Karnataka.

4.9.1.5 Performance of IgM ELISA

Twenty human serum samples were tested with Pan Bio Kit. The positive to sample calibrator ratio was 2.66 and negative to sample calibrator ratio was 0.8. Six samples which were tested positive for IgM antibodies using the coated plates provided in the kit were also positive with the recombinant Lip L32 antigen.

4.9.2 Performance of Dipstick ELISA

By the Dipstick assay and IgG ELISA, similar numbers of serum samples were found to be positive for the dog and human sera from the case patients (Plate 10). None of the sera from healthy individuals were found to be

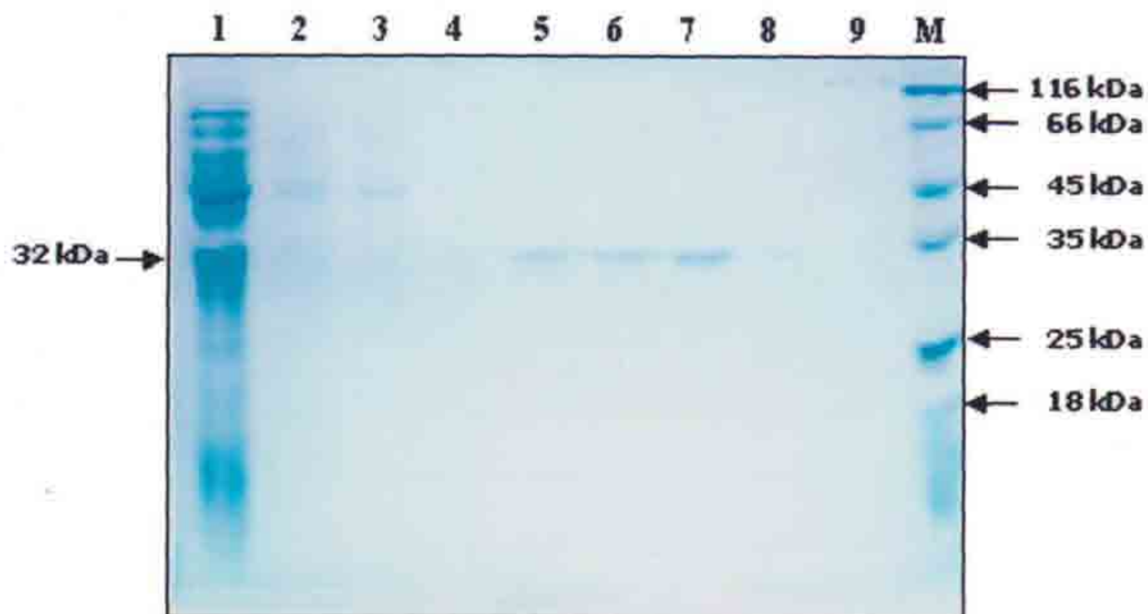


Plate 8 : Purification of recombinant Lip L32 protein by affinity chromatography

Lane 1 : Unpurified recombinant Lip L32 protein
Lane 2 : Eluted protein with 50 mM imidazole
Lane 3 : Eluted protein with 100 mM imidazole
Lane 4 : Eluted protein with 150 mM imidazole
Lane 5 : Eluted protein with 200 mM imidazole
Lane 6 : Eluted protein with 500 mM imidazole
Lane 7 : Eluted protein with 500 mM imidazole
Lane 8 : Eluted protein with 750 mM imidazole
Lane 9 : Eluted protein with 750 mM imidazole
M : Protein Molecular Weight Marker

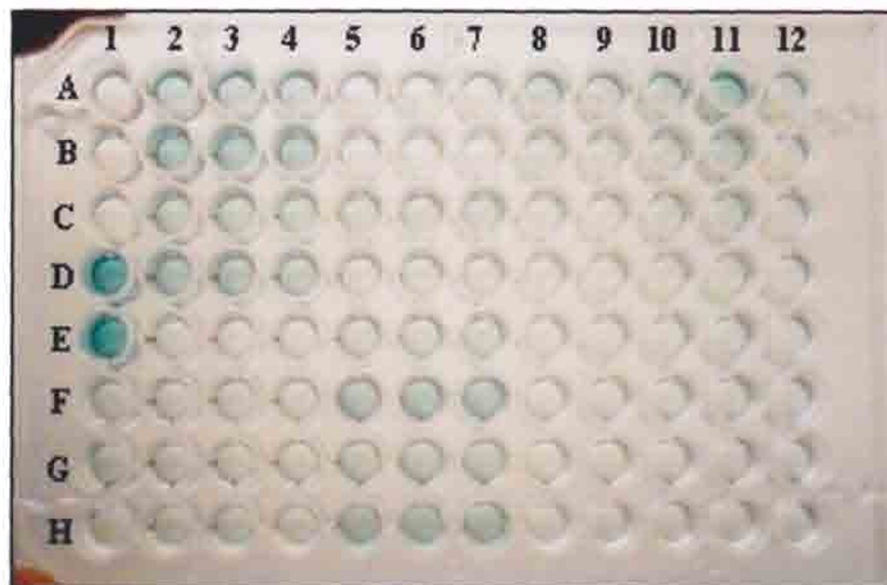


Plate 9 : Recombinant antigen - based single serum dilution ELISA

A1 : Substrate control B1, C1 : HRP conjugate control
D1, E1 : Positive control F1, G1and H1 : Negative control
A2 - H12 : Serum samples diluted at a single working dilution

Table 2

a) Comparison of single dilution IgG ELISA and Microscopic agglutination test in man

		MAT		Total
		Positive	**Negative**	
Single serum dilution ELISA	**Positive**	77	5	82
	Negative	3	51	54
Total		**80**	**56**	**136**

Specificity = 91.07%

Sensitivity = 96.25%

Accuracy = 94.12%

Kappa value = 0.987

b) Interpretation of results

Antibody Titre	Interpretation
0 - 500	Negative
500 - 1000	Suspect
> 1000	Positive

Table 3

a) Comparison of single dilution IgG ELISA and Microscopic agglutination test in dogs

		MAT		Total
		Positive	**Negative**	
Single serum dilution ELISA	**Positive**	60	4	64
	Negative	4	45	49
Total		**64**	**49**	**113**

Specificity = 91.83%

Sensitivity = 93.75%

Accuracy = 92.92%

Kappa value = 0.984

b) Interpretation of results

Antibody Titre	Interpretation
0 - 250	Negative
250 - 500	Suspect
> 500	Positive

positive by either of these assays. The dipstick was coated with an optimum concentration of 25 μg/ml of recombinant Lip L32 antigen. The results of the dipstick assay for positive serum samples were graded from +1 to +4 according to the intensity of the colour reaction (Table 4).

The sensitivity, specificity and accuracy of this assay relative to MAT has been depicted in Tables 5 and 6 for dog and human serum samples, respectively. The kappa values for both tests were 0.977 and 0.971 in dogs and human serum samples respectively, which suggests perfect agreement.

4.9.3 Performance of latex agglutination test (LAT)

A 10% latex suspension was sensitized with 50 μg/ml of the recombinant Lip L32 antigen. By the LAT and Dark Field Microscopy, similar number of serum samples were found to be positive for the dog and human sera from the case patients (Plate 11). Positive results were read on a +1 to +4 scale depending on the extent of agglutination and time taken for the development of agglutination (Table 7). The stability of the latex was determined by testing sensitized latex stored at 4°C and ambient temperature. The stabilized latex beads coated with the recombinant antigen may be stored at 4°C for more than 2 months. At ambient temperature the beads could be stored for one month without showing loss of activity for both weakly and strongly positive samples.

The sensitivity, specificity and accuracy of the test as compared to the standard method is depicted in Tables 8 and 9 for dog and human samples, respectively. Both the tests had a kappa value of 0.981 in dog and human sera respectively, which suggests perfect agreement. The test kit was validated in few laboratories like Leptospirosis Research Laboratory, Madhavaram, Small Animal Clinic, Madras Veterinary College Hospital, Chennai, Institute of Animal Health and Veterinary Biologicals, Bangalore, Karnataka.

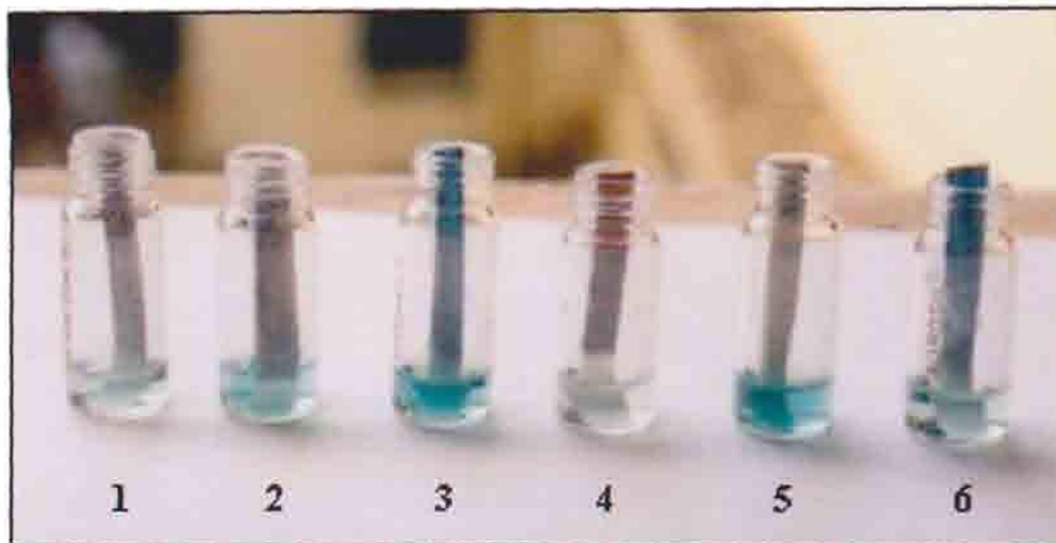


Plate 10 : Recombinant antigen – based Dipstick ELISA

1 : Negative sample
2 : Positive sample (graded + 2)
3 : Positive sample (graded + 3)
4 : Negative control
5 : Positive control (graded + 4)
6 : Positive sample (graded +1)

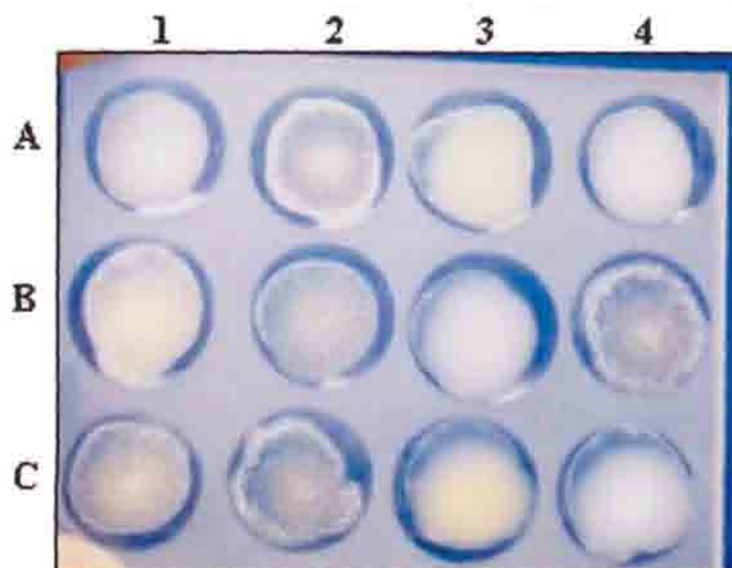


Plate 11 : Recombinant antigen – based Latex agglutination test

A1 : Negative control   A2 : Positive control
B2 : Positive sample graded +1
C1 : Positive sample graded +2
C2 : Positive sample graded +3
B4 : Positive sample graded +4
A3, A4, B1, B3, C3, C4 : Negative samples

Table 4

Dipstick ELISA grading using recombinant Lip L32 antigen in human and canine positive serum samples

Sl.No.	Species	Total	+1	+2	+3	+4
1.	Human	31	18 (58.06)*	6 (19.36)*	6 (19.36)*	1 (3.22)*
2.	Canine	40	26 (65.0)*	9 (22.5)*	3 (7.5)*	2 (5.0)*

* Indicate values in percent

+1 - mild colour in 10-15 min

+2 - moderate colour in 10 min

+3 - dark colour in 5-10 min

+4 - very dark colour - within 5 min

Table 5

Comparison of Dipstick IgG ELISA and Microscopic agglutination test in dogs

		MAT		Total
		Positive	**Negative**	
Dipstick ELISA	**Positive**	38	2	40
	Negative	1	40	41
Total		**39**	**42**	**81**

Specificity = 95.23%

Sensitivity = 97.43%

Accuracy = 96.3%

Kappa value = 0.977

Table 6

Comparison of Dipstick IgG ELISA and Microscopic agglutination test in man

		MAT		Total
		Positive	**Negative**	
Dipstick ELISA	**Positive**	29	2	31
	Negative	1	30	31
Total		**30**	**32**	**62**

Specificity = 93.75%

Sensitivity = 96.67%

Accuracy = 95.16%

Kappa value = 0.971

Table 7

Latex agglutination test grading using recombinant Lip L32 antigen in human and canine positive serum samples

Sl.No.	Species	Total	+1	+2	+3	+4
1.	Human	45	20 (44.44)*	15 (33.33)*	8 (17.79)*	2 (4.44)*
2.	Canine	113	52 (46.02)*	31 (27.43)*	2 (1.77)*	28 (24.78)*

* Indicate values in percent

+1 - mild agglutination in 5 min

+2 - moderate agglutination in 3 min

+3 - heavy agglutination in 2-3 min

+4 - very heavy agglutination in 1 min

Table 8

Comparison of Latex agglutination test and Microscopic agglutination test in dogs

		MAT		**Total**
		Positive	**Negative**	
LAT	**Positive**	51	2	53
	Negative	1	48	49
Total		**52**	**50**	**102**

Specificity = 96%

Sensitivity = 98.07%

Accuracy = 97.05%

Kappa value = 0.981

Table 9

Comparison of Latex agglutination test and Microscopic agglutination test in man

		MAT		Total
		Positive	**Negative**	
LAT	**Positive**	31	2	33
	Negative	2	59	61
Total		**33**	**61**	**94**

Specificity = 96.72%

Sensitivity = 93.93%

Accuracy = 95.74%

Kappa value - 0.981

4.10 Microscopic Agglutination Test (MAT)

In human samples, highest number of positive samples reacted with *autumnalis* followed by *pomona, hebdomadis, grippotyphosa, javanica* whereas in canine samples, highest number of positive samples reacted with *grippotyphosa* followed by *canicola, icterohaemorrhagiae, pomona* and *hebdomadis*.

4.11 Detection of Leptospiral Antigen in Clinical Samples with Recombinant Lip L32 Antigen

4.11.1 Indirect competitive ELISA

By checker board titration, 50 ng per 100 μl of r Lip L32 antigen for coating the plate and antibody at 1:200 dilution was determined for performing the assay.

Using the recombinant Lip L32 as a competitor, the absorbance was lower than the positive control indicating competition to certain extent. However, the clinical samples and the culture when used as the competitor, the colour developed as well as the absorbance was higher than the positive control indicating no competition with the coated antigen.

4.11.2 Indirect competitive Dipstick ELISA

Using the leptospiral culture as the competitor, no colour was visible, indicating nearly 100% competition, where as the positive control (ie, no competitor) indicated 0% competition, as showed by the colour development. But the positive clinical samples indicated 0% competition with the r Lip L32 antigen.

Discussion

5. DISCUSSION

Leptospirosis is now identified as one of the emerging infectious diseases, exemplified by recent large outbreaks in Nicaragua, Brazil, India, Southeast Asia, the United States and Malaysia (Levett, 2001). The diagnosis of leptospirosis cannot be easily made in many laboratories due to the lack of simple diagnostic tools. Hence, leptospirosis is often not recognised or is erroneously mistaken with other diseases of similar symptoms. As a consequence, this serious disease may be either left untreated or treated improperly; besides, information on the prevalence and incidence of leptospirosis may be unreliable. The MAT is the standard serologic test for diagnosis of leptospirosis. The test requires dark field microscopy and the laboratory must maintain cultures of the various serovars tested. Thus, this test usually is referred to a commercial laboratory. The classic confirmation of leptospiral infection is based on a four fold rise in the MAT titre. Moreover, acute titres drawn within the first 7 to 10 days of infection may be negative. Thus, it requires analysis of paired sera to achieve sufficient sensitivity (Cumberland *et al.*, 1999). Vaccination causes serovar specific titres that are rarely greater than 1:300 and high vaccinal titres rarely persist for more than 3 months after vaccination (Bolin, 1996).

Several ELISA tests have been developed based on whole cell leptospiral antigen preparations. This warrants the development of an improved diagnostic test which can detect the organism in the initial phase of the disease. Recombinant antigen based serologic tests may achieve high sensitivity and specificity than other tests because of the purity of the immunodominant antigen and lack of non-specific moieties present in whole - cell preparations.

The serovar *pomona* strain Pomona of *L. interrogans* species was chosen for cloning and expression of the recombinant outer membrane protein. The serovar *pomona* is a broadly reactive antigen as it is prevalent in canine leptospirosis (Langston and Heuter, 2003), bovine leptospirosis (Cousins *et al.*, 1985) and human leptospirosis (Gussenhoven *et al.*, 1997).

5.1 DNA Extraction

The high salt method as described by Lahiri and Nurunberger (1991) and Senthilkumar and Ramadass, 2001 was slightly modified for extraction of leptospiral DNA. This is achieved by salting out the cellular proteins by dehydration and precipitation with a saturated sodium chloride solution. It also eliminates the step of prolonged digestion of samples with proteinase K and avoids the use of phenol, chloroform and isoamylalcohol thus, saving the cost and time of operation. The DNA obtained is of good quality. This method is rapid and the DNA is suitable for PCR analysis.

5.2 Polymerase Chain Reaction for the Amplification of Partial Lip L32 Gene

The forward and reverse primers for amplifying a portion of Lip L32 gene (Plate 1) were designed based on the published Lip L32 gene sequence of *L. kirschneri* species (Haake *et al.*, 2000). The restriction enzyme sites for *Xba*I and *Kpn*I were incorporated at the 5'ends of the primers for cloning in the expression vector as sites for *Xho*I and *Sma*I were present in the primers for amplifying Lip L32 gene of *L. kirschneri* species (Haake *et al.*,

2000). Moreover, the primers developed in this study are able to amplify only the pathogenic serovars and not non-pathogenic serovars of *Leptospira* species (Plate 1). This finding supports that the sequence and expression of Lip L32 is confined to the pathogenic *Leptospira* species (Haake *et al.,* 2000).

5.3 Cloning of the Lip L32 Gene into pBluescript II KS (+) Vector

The PCR - amplified partial Lip L32 gene is cloned into pBluescript II KS (+) cloning vector for sequencing. Blunt end cloning was followed by ligating the *Sma*I digested plasmid to the T4 DNA polymerase filled PCR fragment. Due to blunt end ligation, only 2 were recombinant clones and the correct orientation of the insert was checked by colony PCR using vector specific primer T3 as the forward primer and the reverse gene specific primer (Plate 3). The restriction enzyme digestion with *Eco*RI/*Kpn*I also confirmed the presence of insert in the recombinant plasmid (Plate 3). The insert which is 700 bp will only be released if the gene has been cloned upstream in the cloning vector. Similar cloning strategy was followed by Heikkila *et al.* (2002) where the BBK 32 ampified gene was cloned into the PCR 2.1 - TOPO plasmid. The recombinant plasmid was then digested and the cleaved BBK 32 was then ligated to a similar digested pGEX-4T-1 expression plasmid.

5.4 Sequence Analysis

The target genes chosen to be indicators of relatedness for phylogeny should fulfill that they should either be essential elements in all the microorganisms studied or have a conserved function in order that relatedness

should be assessed. Bacteria have larger genomes than viruses and code for many more proteins and structural and regulatory molecules. As it is not yet practical to determine whole genome sequences of bacteria for phylogenetic purposes, relatively conserved genes are used to compare and classify bacteria.

The principles involved in the analysis of phylogenetic relationships between isolates are similar, whether the data consist of nucleotide sequences, restriction fragments or amino acid sequences. The first approach is based on a distance matrix generated from all pairwise comparisons of DNA sequences. The phylogeny can then be inferred by applying the neighbour joining method because it is extremely fast and highly efficient at finding the correct tree. Hence, the NJ method (Saitou and Nei, 1987) was used in getting the phylogenetic tree.

In this study, the Lip L32 structural gene of serovar *pomona* strain Pomona consists of 665 bp encoding a protein of 221 amino acids and remaining around 45 amino acids from the residues of the fusion tag (Fig. 1). The portion of structural gene of Lip L32 cloned and sequenced in this study was submitted to GenBank under the accession number AY223718. The portion of the structural gene that it aligned with the other *Leptospira* species consists of 639 bp encoding for a protein of 213 amino acids (Figs.2 and 3).

The Lip L32 sequence is consistent with previous findings (Haake *et al.*, 2000) and is highly conserved with an average DNA sequence similarity of 97.42% with other strains (Table 1). Another interesting finding is that the expression of the Lip L32 protein is confined only to the pathogenic serovars of *Leptospira*. This finding is in agreement with the finding of Haake *et al.* (2000).

The phylogenetic tree of Lip L32 represents three groups (Fig.3) with four genome species of Leptospira namely *interrogans, kirschneri, borgpetersenii* and *santarosai*. The serovars of *L. interrogans* namely *pomona, australis, canicola, icterohaemorrhagiae* and *autumnalis* were related and formed a cluster. Among these serovars, the strains Pomona and RZ11 of the serovar *pomona* had no difference. The serovar *grippotyphosa* of *L. kirschneri* and serovars of *L. interrogans* formed one monophyletic group as supported by Haake *et al.* (2000).

The amino acid sequence of the serovar *pomona* strain Pomona was conceptually translated and aligned with other predicted amino acid sequences of other *Leptospira* species (Fig.4). The comparison of the deduced amino acid sequences of the Lip L32 variants revealed an average amino acid sequence identity of 98.06%. Most of the sequence polymorphisms detected (39 of 56) were silent as supported by Haake *et al.* (2000). The I-T-A-C lipoprotein signal peptidase cleavage site is similar to another lipoprotein OMP Lip L36 which has L-T-A-C signal peptidase cleavage site. An isoleucine at the -3 position relative to cysteine is less common than leucine in signal peptidase II cleavage sites, but there are many other examples of isoleucine occurring at this position in bacterial lipoproteins including OspF, Erp G, BBA 59, and BBA 60 of *B.burgdorferi*, Env C and Exc C of *E.coli* and Lpp 20 of *Helicobacter pylori*. Beginning at residue 150 of the Lip L32 in this study, revealed an unusual cluster of a series of aspartate residues in a span of eight amino acids, a finding reminiscent of the six-aspartate cluster of Lip L36 (Haake *et al.*, 1998).

5.5 *In-vitro* Expression of Lip L32 Protein

The expression of heterologous genes coding for protein of interest in an appropriate system of expression has emerged as a powerful tool for large scale production of protein for diagnostics and immunoprophylactic purposes. *Escherichia coli* is the most favoured organism for expression of many protein. Ease of genetic manipulation, high rates of cell growth and low cultural conditions make *E.coli* a popular system for gene expression. It has now been possible to produce hybrid protein through *in vitro* gene fusion which is highly practicable and preferred method for generating recombinant antigens, because of the high degree of certainty of expression.

The outer membrane of leptospires contain lipopolysaccharides (LPS) and several lipoproteins (OMPs). The LPS is highly immunogenic and is responsible for serovar specificity (Levett, 2001). Several of the leptospiral OMPs are highly conserved (Haake *et al.,* 2000). As a result, the major outer membrane protein Lip L32 was used in this study for the development of new approaches for the serological diagnosis of leptospirosis.

There are two general strategies for expressing recombinant proteins to be used as antigens. The first involves the in frame fusion of all or part of the DNA encoding the protein of interest to bacterial coding sequences. The second strategy involves expressing the foreign gene directly in vectors providing very high level inducible prokaryotic promoter and a leader sequence for efficient translation initiation to get stable protein. In this study, the portion

of the Lip L32 gene was inframe with the 'a' series of the expression vector pPro Ex HT. The orientation of the insert was checked by colony PCR (Plate 4) and restriction enzyme analysis (Plate 5). The recombinant clone designated as pPro10 showed high level of expression under trc promoter of the vector when induced with IPTG. The protein kinetics study showed that maximum level of expression was between the second and third hours interval post-induction (Plate 6).

5.6 Characterization of the Recombinant Lip L32 Protein

IgG Western Blot assays with recombinant Lip L32 as the antigen was performed as preliminary serologic analysis. The hyperimmune mice serum against the serovar *pomona* reacted with the antigen showing a single band. The dog and human positive serum samples also reacted with the recombinant Lip L32 antigen. But the antigen did not react with the rabbit hyperimmune serum against the serovar *patoc*. The conclusion that Lip L32 is expressed among the pathogenic serovars is consistent with previous findings of Haake *et al.* 2000. In this study, the molecular mass of 32 kDa is due to the rLip L32 protein as well as the amino acid residues from the expression vector (Plate 7).

5.7 Purification of the Recombinant Lip L32 Protein

The recombinant protein is expressed as a fusion protein tagged with polyhistidine to the 5'end. This peptide tag was exploited for purification of the recombinant Lip L32 using nickel by affinity chromatography. The outer

membrane proteins formed inclusion bodies in the cytosol and the recombinant fusion protein is solubilized with 6 M guanidine (Flannery *et al.*, 2001). In this study, 8 M urea was used to solubilize the culture pellet. The advantage of using urea is that the eluted protein can be directly loaded in SDS - PAGE whereas the fractions that contain guanidine hydrochloride will form a precipitate when treated with SDS. They must either be diluted with water, dialyzed before analysis or separated from GU-HCl by trichloroacetic acid precipitation. Imidazole at concentrations 50 mM to 750 mM were tried to elute the 6 X His-tagged protein from the nickel column. In this study, maximum protein was eluted at 500 mM concentration of imidazole which binds and competes with histidine residues in the 6 X His tag of the recombinant protein (Plate 8).

5.8 Detection of Leptospiral Antibodies in Clinical Samples by Recombinant Lip L32 Antigen

The first aim of this study was to develop an ideal recombinant antigen and this has been achieved. An ideal antigen would be a principal target of the host immune response, expressed only in pathogenic *Leptospira* species and conserved among more than 200 serovars associated with leptospirosis in different geographical regions and epidemiological situations. An ideal test need to discriminate between leptospirosis and a broad spectrum of diseases that cause acute febrile illnesses and have overlapping clinical presentations.

5.8.1 Enzyme-linked immunosorbent assay (ELISA)

A rapid, accurate method for the diagnosis of leptospirosis is important to both the clinician and the patient. Direct detection of *Leptospira* by microscopic examination or culture is impractical due to low success rate and the amount of time and labour required. The MAT however, despite its wide spread usage and international recognition, has a number of limitations. These include the need to use hazardous live bacteria, time consuming and expertise to test each serum sample against multiple serovars of this organism. In this study, the conserved nature and high level of expression of Lip L32 only among the pathogenic *Leptospira* species suggested that the rLip L32 ELISA may exhibit similar performance regardless of the locally predominant serovar.

The recombinant leptospiral protein Lip L32 was evaluated as antigen in ELISA. The maximum difference in the positive and negative sample absorbance values were observed at a concentration of 50 ng/well (Fig.5) whereas maximum absorbance values were observed at 25 ng/well in the rLip L32 ELISA as reported by Flannery *et al.* (2001).

In this study for the first time, we took the advantage of the single serum dilution ELISA (Plate 9) by establishing a nearly linear relationship between the corrected titre of antisera at a single working dilution and the corresponding observed antibody titire (Figs.8 and 9) as reported for the detection of antibodies against the Newcastle disease virus (Snyder *et al.,* 1983). The use of single serum dilution reduces the reagent costs and technical time

significantly, and may reduce error inherent in serial dilutions. In this method the PNT baseline was arbitrarily set at an absorbance by plotting the average of triplicate, corrected absorbance readings at serial dilutions of the observed titres for positive and negative sera. In dog samples (Fig.6) indicates that a dilution of 1:500 ($\log_{10}$ value = 2.7) and Fig.7 in human samples, which indicates that at a dilution of 1:1000 ($\log_{10}$ value = 3) and above, the absorbance of the negative sera plateaued whereas, the positive sera continued to show a high absorbance value. Thus the results were interpreted, where in human serum samples (Table 2) as the antibody titres $\geq$ 1000 were considered positive, between 500 - 1000 as suspected where the serum samples should be retested and below 500 as negative. In dog serum samples, the results were interpreted (Table 3) as the antibody titre $\geq$ 500 were considered positive, between 250 and 500 as suspected and below 250 as negative. The S/P ratio is used instead of the OD values at a particular dilution for standardization of single dilution ELISA since a great variation in OD values for a particular sample is observed depending upon the assay conditions, time of incubation etc. However, the S/P ratio for a particular serum sample at the same dilution remains relatively constant under varying test conditions. Both human and canine serum samples of known identity were deliberately selected from a previous study for the standardization of serial and single dilution ELISA to increase the confidence of the regression equation so that unknown sera with different antibody titres could be evaluated perfectly in this system. This was in conformity with earlier studies of Briggs and Skeels (1984), Manuja *et al.* (2001) who adopted a similar strategy while standardising single dilution ELISA for detecting antibodies to other pathogens.

A relatively high sensitivity and specificity between ELISA and MAT titres (Tables 2 and 3) is a good indication that the tests measure similar trends in exposure to leptospirosis. The kappa value was almost 1.0 indicating perfect agreement. The ELISA had a specificity of 91.07% and 91.83% in human and dog sera respectively relative to the MAT. This relative specificity value, which was obtained with sera defined by the MAT, may contain non-agglutinating leptospiral antibodies that are detectable by ELISA but not by the MAT, which can only detect agglutinating antibodies as reported previously by Ribotta *et al.* (2000). Few samples collected from MAT confirmed leptospirosis cases that had reciprocal MAT titres greater than or equal to 100 had negative recombinant Lip L32 ELISA result. The low background reactivity may be due, in part, to the restricted expression of Lip L32 in pathogenic leptospires (Haake *et al.,* 2000) and not saprophytic forms that are ubiquitous in the environment.

Performance of the recombinant Lip L32 (rLip L32) antigen with the commercially available PanBio IgM ELISA revealed that the rLip L32 detected IgM response. This finding is contradictory to the findings reported by Flannery *et al.* (2001). The predominant humoral response during acute phase infection is believed to be due to IgM antibodies (Adler *et al.,* 1980), which are directed primarily against carbohydrate epitopes. The IgG response to rLip L32 and other recombinant antigens were found to have kinetics comparable to that of the IgM response to whole - antigen preparations (Flannery *et al.,* 2001). They further explained that the rapid rise in IgG antibody may have been due to a memory response in individuals with prior exposure to leptospires. Alternatively, this could represent a rapid IgM to IgG class switch phenomenon.

These findings suggest that early host immune response to Leptospira infection is characterized by both IgM and IgG antibodies specific for different moieties, as observed in the early response to Borrelia (Heikkila *et al.,* 2002). Moreover, in this study few human serum samples showed high IgM as well as IgG titres and this finding is supported by Terpstra *et al.* (1985a) where characteristically high IgM, IgG and agglutinating titres were found only in patients with current leptospirosis.

The recombinant Lip L32 ELISA, in this study also differentiated patients with leptospirosis from those with other important causes of acute jaundice and febrile illnesses, such as typhoid, since the serum samples positive for typhoid and hepatitis did not show any reaction with the recombinant protein which corraborates the findings of Flannery *et al.* (2001). Due to the many advantages of rLip L32 single serum dilution ELISA, such as its excellent diagnostic specificity and sensitivity, convenient technical features, its potential for automation, the test may be efficiently utilized as screening test for large number of serum samples (Plate 9) for the detection of leptospiral antibodies.

5.9.2 Dipstick ELISA

Enzyme linked immunosorbent assays are useful alternative or additional tests by viture of their capacity to detect with a high sensitivity, specific antibodies as a cause of leptospirosis. Because of the relative complexity and costs of these assays, they can only be performed in relatively specialised laboratories, thus hampering the diagnosis and appropriate treatment of leptospirosis in many tropical countries (Gussenhoven *et al.,* 1997). A

simplified version of the ELISA giving either a positive or a negative indicative result would be useful as a screening test for detecting leptospiral antibodies in situations where appropriate diagnostic facilities are lacking. Hence, in this study, we have developed a simple dipstick method (Plate 10) for the detection of Leptospira - specific IgG antibodies in both human and dog serum samples using the recombinant Lip L32 antigen (rLip L32). Flannery *et al.* (2001) believed that rLip L32 could be incorporated in rapid formats, such as dipstick, to facilitate its use in serodiagnosis in developing countries.

Evaluation of the assay revealed that the results of the assay are in agreement with the results of the MAT - confirmed leptospirosis cases. Moreover, nearly the same number of serum samples from patients and controls were found to be positive by the dipstick assay as by the IgG ELISA. This shows that the method is well suited for the diagnosis of leptospirosis. The dipstick method has few advantages, that it is easy to perform, is highly reproducible, and depends on neither special equipment nor refrigeration.

The results of the dipstick assay for the positive serum samples were rated 1+ to 4+ according to the intensity of colour development in the substrate solution (Table 4). In the dipstick assay, most of the positive sera from the MAT confirmed leptospirosis cases revealed a high colour development in both human and dog sera samples. The assay corresponds well with the MAT in terms of sensitivity and specificity in both the human (Table 5) and dog (Table 6) serum samples. The test showed specificity of 95.25% in dogs and 93.75% in man. The sensitivity was 97.43% in dogs and 96.67% in man, and the kappa value was 0.97 for both dog and human serum samples, which shows perfect agreement.

We have developed an easy to perform dipstick method for use by clinical and field laboratories for the first time as a valuable addition to the armaments for the serodiagnosis of canine and human leptospirosis. The assay is performed by coating the strips with pure rLip L32 antigen at 25 μg/ml and preparing a 1:200 single dilution of serum. Hence, the numerous practical advantages of the dipstick assay may well contribute to an improved diagnosis of leptospirosis.

5.9.3 Latex agglutination test

A rapid, new latex agglutination assay was developed to detect the leptospiral antibodies from human and animal samples. In the present study, the latex beads were sensitized with the recombinant Lip L32 antigen. Thus, the coated beads could detect the antibodies against the pathogenic serovars of *Leptospira* species. This is the first work that reports the diagnosis of pathogenic *Leptospira* using the recombinant outer membrane protein by LAT (Plate 11). The test is extremely simple and inexpensive, at the same time requires no specific expertise and expensive or sophisticated equipment. It is extremely rapid (2-5 min) and sensitive in detecting the antibodies produced against the pathogenic *Leptospira*. Another advantage of this assay is that the same coated beads could be used to screen both human and animal samples without any other reagents.

Muraschi, as early as 1959 reported LAT for the detection of leptospirosis, but the antigens were not stabilized. Later, Ramadass *et al.* (1999) prepared sonicated antigen from the serovars *icterohaemorrhagiae, autumnalis, australis* and *canicola* and coated the latex beads with an equal volume of

pooled leptospiral antigen. Smits *et al.* (2000) used heat stable antigen for conducting LAT but it gave very less positivity when a set of known high positive and negative sera were screened. Verma (2001) in his study showed that sonicated antigen gave better results than the heat stable antigen. He prepared sonicated antigen from serovars *pomona* and *hardjo*. These serovars were selected on the basis of ELISA results which showed highest positivity by *pomona* and better titre by *hardjo*. Moreover, serovar *pomona* is reported to be an emerging serovar in many parts of the world (Ribotta *et al.,* 2000). However, in this study, the purified recombinant lipoprotein Lip L32 which is highly conserved in all the pathogenic serovars was developed to sensitize the latex beads at a concentration of 50 μg/ml in carbonate bicarbonate buffer (pH 9.6).

Traditionally, diagnosis of leptospirosis depends on the demonstration of leptospires in clinical samples by dark field microscopy (DFM), by demonstration of leptospiral antibodies by MAT or ELISA or by culturing of leptospires. Detection of leptospires in blood and urine by DFM is not always possible and the possibilities of false positive results are greater with inexperienced workers. In this study, nearly equal number of serum samples of both human and dog, were found to be positive by both DFM and LAT.

The stability of the latex was determined by storing the sensitized latex beads at 4°C and ambient temperature. The coated beads could be stored at 4°C for more than 3 months and at ambient temperature for a month without showing loss of activity of both weekly and strongly positive samples. The assay was performed by mixing 15-20 μl of serum sample with an equal amount

of sensitized beads and the agglutination was recorded with naked eye. The samples were considered negative if no agglutination was observed within 5 min and positive results were read on a +1 to +4 scale depending on the extent of agglutination and time taken for the development of agglutination (Table 7).

In leptospirosis, antibodies usually appear within 5 to 7 days after the onset of the symptoms and in a significant proportion of patients, antibodies persist in detectable quantities for many months (Faine, 1982). IgM antibody levels increases in the first week, but become negative within 3-5 weeks whereas IgG antibody is detected at about the same time as IgM, but persists for a much longer period (Cousins *et al.,* 1985).

In this study, few dog serum samples which were graded as +4 in LAT were also tested for recombinant Lip L32 IgG ELISA. In ELISA those same samples had an IgG titre below the cut off value. Moreover, the organisms were detected in those serum samples under DFM. The history of those dogs and their symptoms revealed acute infection. Similar case study in human patients, showed high IgM titre and low IgG titre and the serum samples were graded as +4 in LAT. This is consistent with the findings of Terpstra *et al.* (1985a) who reported high IgM titres between 10 and 60 days of illness while the combination of moderately increased IgM titres and low IgG titres were most often observed in the first 10 days of the disease. Moreover, the results reported by Smits *et al.* (2000) indicated that reactivity in the latex assay corresponds with the presence of specific IgM antibodies.

The sensitivity and specificity of LAT relative to MAT were calculated. One MAT positive serum sample was negative by LAT in dogs (Table 8) as a result the sensitivity was 98.07% whereas two MAT negative samples were positive by LAT, as a result specificity was 96%. On the other hand, the sensitivity and specificity with human serum samples (Table 9) were 93.93% and 96.72% respectively. The sensitivity was 93.8% and specificity 94.9% for human serum samples collected 10 to 30 days after their onset of the disease (Smits *et al.,* 2000). The kappa value was 0.981 for both dog and human serum samples and according to the guidelines for interpretation of 'k' value any value greater than 0.81 indicates perfect agreement.

Leptospirosis affects mostly those living in tropical countries who cannot rely on hospital facilities with laboratory support. This assay is extremely simple and rapid, does not require training to perform the test and results are concordant with those of the MAT even for those samples collected early in the disease. The test can be performed in situations where facilities or resources to perform more complicated tests are not available. As the assay is rapid, it has a role in the management of patients, especially when attention must be given to a large number of patients (Smits *et al.,* 2000).

5.7 Microscopic Agglutination Test

The standard serologic test, the microscopic agglutination test (MAT) was performed for both human and dog serum samples. Confirmed positive and negative serum samples were also obtained from the Leptospirosis Research Laboratory, Chennai. A MAT titre of $\geq$ 100 was selected as indicative of an active leptospiral infection. In this study, the results of the immunoassays ELISA, Dipstick ELISA and LAT were expressed relative to those obtained with the internationally recognized MAT. In human samples, the highest number

of positive samples reacted with *autumnalis* followed by *pomona, hebdomadis, grippotyphosa, javanica* whereas in canine samples highest number of positive samples reacted with *grippotyphosa* followed by *canicola, icterohaemorrhagiae, pomona* and *hebdomadis*. The results were in partial agreement with previous findings of Ratnam *et al.* (1994) who showed that amongst dog population *canicola, autumnalis* and *pomona* were the three commonest prevalent serogroups. Ratnam *et al.* (1994) also reported that *pomona* and *autumnalis* were prevalent serovars amongst human population in the Southern and Northern districts of Tamil Nadu, respectively.

Despite its widespread usage and international recognition, MAT is inadequate for rapid case identification since it can only be performed in a few reference laboratories and requires analysis of paired sera to achieve sufficient sensitivity (Cumberland *et al.,* 1999). Moreover, dependence upon the MAT results in delays in establishing the cause of outbreaks, as seen in several investigations (Flanney *et al.,* 2001).

5.10 Detection of Leptospiral Antigen in Clinical Samples with Recombinant Lip L32 Antigen by Indirect competitive assay

An attempt was made to detect the leptospiral antigen in clinical samples using recombinant Lip L32 antigen (rLip L32). The indirect competitive ELISA was tried first and by checker board titration the concentration of antigen and the dilution of antibody were determined. Indirect competitive ELISA was performed to detect the antigen in the clinical samples. But the antigen could not be detected in the clinical samples as the antigen in samples were too low to compete with the rLip L32 antigen. So, rLip L32 as a competitor antigen was tried and competition could be detected.

As competitive ELISA did not show satisfactory results the indirect competitive Dipstick ELISA was carried out. Here, the test was standardized according to the principle of the assay. The leptospiral culture as a competitor showed 100% competition with the rLip L32 whereas the positive serum samples (without any competitor) showed 0% competition. But the clinical samples showed no appropriate results. The reason may be low concentration of antigen in clinical sample. Crowther (1995) described that competition only occurs where extremely high concentration of competitor are used. Since, no other work was there, it was difficult to compare and discuss the results.

There is an urgent need for development of new serodiagnostic strategies for leptospirosis which can detect the infection in the initial stage of illness. Antibodies are detectable in the blood approximately 5 to 7 days after the onset of symptoms (Levett, 2001). Effective tests that could allow detection of organisms could provide a diagnosis earlier in the course of disease but are less reliable (Langston and Heuter, 2003). In this study, the recombinant antigen based latex agglutination test could detect the recent exposure of leptospirosis.

5.11 Conclusion

The first aim of this study was to develop a recombinant antigen for the diagnosis of leptospirosis and has been achieved. The portion of Lip L32 gene from the serovar *pomona* was amplified with the primers which were designed in this study. The primers were specific for the pathogenic *Leptospira* species and the non-pathogenic leptospires could not be amplified. The portion of Lip L32 amplicon from serovar *pomona* was cloned in pBluescript II KS (+) vector and the recombinant plasmid pKSP4 was sequenced.

The nucleotide and amino acid sequence of the cloned Lip L32 were aligned and compared with other available Lip L32 sequences. Lip L32 showed high degree of conservation in pathogenic leptospires.

The Lip L32 amplicon with built-in restriction sites were utilized for directional cloning in pPro EX HT 'a' expression vector. The recombinant clone pPro10 was selected by colony PCR and restriction enzyme analysis.

The recombinant clone pPro10 transformed in DH5α cells were induced with 1 mM IPTG. The protein kinetics study showed maximum expression during the second and third hours interval post induction.

The specificity of the protein was determined by immunoblot analysis and a specific single band of 32 kDa was obtained when probed with pathogenic leptospiral antibodies. The recombinant antigen could not react with serum against non-pathogenic serovar *patoc*. This explains that the Lip L32 expression is confined to the pathogenic serovars of *Leptospira*.

The Lip L32 is expressed as a fusion protein, tagged with polyhistidine residues. The 8 M urea solubilized the protein and purification was done by affinity chromatography.

Improved diagnostic tests like single serum dilution ELISA, Dipstick ELISA and Latex agglutination test were developed based on the recombinant Lip L32. The tests developed had excellent specificity and sensitivity relative to microscopic agglutination test. Among these diagnostic tools, LAT is extremely simple and rapid, could be performed in situations where facilities for

complex tests are not available. The sensitized beads had a shelf-life upto one month when stored at ambient temperature. Moreover, the sensitized beads could detect the leptospiral antibodies in the initial stage of illness.

Attempts were made to investigate the possibility of detecting leptospiral antigen in clinical samples by indirect competitive assay. But the low concentration of antigen in clinical samples could not compete with the recombinant Lip L32 as a result, no satisfactory results were obtained.

The recombinant Lip L32 developed in this study could be used to diagnose Leptospirosis at an early stage of illness, caused by pathogenic serovars of *Leptospira*. The results obtained from the serum samples were concordant with those of the standard method, MAT.

Summary

6. SUMMARY

Leptospirosis is an important disease of animals and human beings caused by pathogenic *Leptospira* species. In the present study, the serovar *pomona* strain Pomona of the pathogenic *Leptospira interrogans* was chosen for developing the recombinant outer membrane protein Lip L32. The recombinant Lip L32 protein was used as a tool for the diagnosis of leptospirosis.

Primers have been designed with built-in restriction enzyme sites for amplification of a portion of Lip L32 gene of the pathogenic serovars of *Leptospira* species. This pair of primers were able to differentiate the disease causing pathogenic from the saprophytic non-pathogenic *Leptospira* species. Use of the designed primers would amplify a 665 bp Lip L32 gene and favour directional cloning of the amplified gene.

The PCR amplified Lip L32 gene had been cloned into the pBluescript II KS (+) cloning vector by blunt end cloning and transformed into the DH5α cells. Screening of the colonies by colony PCR yielded recombinant clone designated as pKSP4. The orientation of the insert was checked by using vector specific primer T3 as forward and reverse gene specific primer. The restriction enzyme digestion with *Eco*RI/*Kpn*I further confirmed the presence of insert in the recombinant plasmid. The recombinant plasmid pKSP4 was sequenced and the sequence of the Lip L32 gene was submitted to GenBank under the accession number AY223718.

The conceptually translated amino acid sequence revealed only one long open reading frame with the signal peptidase cleavage site II, I-T-A-C at the beginning of the sequence. Programmes predicted the other characteristics of the derived amino acid sequence, which coincided well with the earlier reports. The sequence and expression of Lip L32 is highly conserved among the pathogenic *Leptospira* species.

By directional cloning, the Lip L32 was subcloned into the pPro EX HT 'a' expression vector. The recombinant clone designated as pPro10 showed high level of expression under trc promoter of the vector when induced with IPTG. The protein kinetics study showed that maximum level of expression was during the second and third hours interval post induction.

To addresses the level and distribution of Lip L32 expression, immunoblot analysis was performed using antiserum from mice immunized with serovar *pomona* and a rabbit immunized with serovar *patoc*. The antiserum raised against serovar *pomona* was reactive with a single band of 32 kDa, even the positive human and dog serum samples reacted with the recombinant Lip L32. No recombinant Lip L32 was detected when reacted with the non-pathogenic *Leptospira* species, serovar *patoc*.

The recombinant protein with the polyhistidine tag was solubilized with 8 M urea and the fusion protein was purified by affinity chromatography. 500 mM imidazole eluted out the maximum amount of protein. The concentration of the recombinant protein was determined.

Recombinant antigen - based single serum dilution ELISA was developed with many advantages such as excellent diagnostic specificity and sensitivity and the test could be efficiently utilized as screening test for large number of dog and human serum samples for the detection of leptospiral antibodies.

An easy-to-perform recombinant antigen-based dipstick ELISA was developed for use by clinical and field laboratories as a valuable addition to the armaments for the serodiagnosis of leptospirosis.

A simple and rapid latex agglutination test was developed using the recombinant Lip L32 antigen to detect the leptospiral antibodies from human and animal samples. The test could be performed to detect the leptospiral antibodies in the initial phase of the disease. As a result the treatment could be initiated soon which could prevent severe complications and death.

Attempts were made to investigate the possibility of using the recombinant antigen for detecting the antigen in the clinical samples by Indirect competitive assay. Both ELISA and Dipstick methods were tried but as the concentration of the antigen in the clinical samples is less no satisfactory results were obtained.

In many developing countries where leptospirosis is an endemic problem and poses a serious threat to both human and animal populations, rapid diagnosis of the disease at an early stage, is the need of the hour. The recombinant Lip L32 protein which has been developed in this study would satisfy the above said criteria thereby saving precious human life and considerable livestock economy.

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