Studies on the hemin-binding protein HbpA and the iron-regulated virulence factor sphingomyelinase in pathogenic leptospires

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By

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Studies on the hemin-binding protein HbpA and iron-regulated virulence factor sphingomyelinase in pathogenic leptospires**" is the result of the investigation carried out by me in the Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad. As a recipient of JRF / SRF by the Council of Scientific and Industrial Research (CSIR), the present study was done under the supervision of **Prof. Manjula Sritharan.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators. Any omission, which might have occurred by oversight or error, is regretted.

March 2009 **SRIDHAR VELINENI**

Statement verified

Prof. Manjula Sritharan
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CERTIFICATE

Certified that the work embodied in this thesis entitled "**Studies on the hemin-binding protein HbpA and the iron-regulated virulence factor sphingomyelinase in pathogenic leptospires**" has been carried out by **Mr. Sridhar Velineni** under my supervision and the same has not been submitted elsewhere for a degree.

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Abbreviations

BLAST	: Basic Local Alignment Search Tool
Bp	: Base pair
CDS	: Coding sequences
DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic acid
EDDHPA	: Ethylenediamine di-o-hydroxyphenylacetic acid
FepA	: Ferric - enterobactin receptor protein
Fur	: Ferric - uptake regulator
H	: Hour
IRPs	: Iron-regulated proteins
Kb	: Kilobase pair
KDa	: Kilodalton
KV	: Kilo volt
LPS	: Lipopolysaccharides
MEGA	: Molecular Evolutionary Genetics Analysis
µM	: Micromolar
mg	: Milligram
mL	: Milliliter
mM	: Millimolar
NCBI	: National Center for Biotechnology Information
nm	: Nanometers
OMP	: Outer membrane protein
OMV	: Outer membrane vesicle
O/N	: Over night
PCR	: Polymerase chain reaction
Sph	: Sphingomyelinase
Tris	: Tris - (Hydroxymethyl) aminoethane
TAE	: Tris-Acetate-EDTA buffer.
UV	: Ultra violet

Abstract

Leptospirosis is a spirochetal zoonosis of worldwide distribution. It is caused by the pathogenic members of the genus *Leptospira* that includes more than 250 different serovars, whose distribution may be restricted geographically. Despite the high antigenicity of surface lipopolysaccharide molecules, they afford little cross-protection against infectivity by other serovars. In addition, an economic and easy-to do specific diagnostic test is necessary for early diagnosis. Thus candidate antigens are to be identified both for diagnosis and vaccine purposes. This can be achieved by a better understanding of host-pathogen interactions. Acquisition of iron is one of the important factors contributing to the successful establishment of the pathogen, as the amount of available iron is limiting in the mammalian host. Though the requirement of iron by *Leptospira* sp. is known, there are no reports on the mechanism of iron acquisition. Earlier studies in our lab showed for the first time that pathogenic leptospires acquire iron directly via a hemin receptor. Siderophores could not be detected in the spent growth medium. Using a bioinformatics approach, a TonB-dependent outer membrane protein (LB191) was identified in *Leptospira interrogans* serovar Lai using the Fe^{3+}-enterochelin receptor FepA of *Escherichia coli*. The protein, referred to as HbpA showed a similar fold as other Fe^{3+}-siderophore receptors upon homology modeling. Wet lab experiments with recombinant HbpA confirmed it to be a hemin receptor.

In this study, after several trials with different chelators, we established conditions of iron-deprivation for the growth of *Leptospira* spp. with EDDA at 200 µM final concentration, with a final incubation at 37ºC for 5½ h. We detected HbpA as an 81 kDa protein in low-iron organisms of *L. interrogans* serovar Lai that was not expressed in high-iron conditions. Analysis of several leptospiral species showed that HbpA was restricted to serovars belonging to *L. interrogans*. Immunofluorescence studies using anti-HbpA antibodies demonstrated the surface expression of HbpA by iron-limited leptospires. Incidentally, a constitutively expressed 44-kDa protein was identified as a hemin-binding protein and identified as LipL41, both by sequence analysis and immunoblotting studies with specific anti-LipL41 antibodies. The diagnostic potential of HbpA was evaluated both by immunological and molecular methods. Antibody-based detection using ELISA was used to screen serum samples from patients with

leptospirosis. The specificity and sensitivity of the ELISA, calculated using MAT as standard was shown to be 87.18% and 81.81% respectively. PCR of reference serovars of *Leptospira* spp. showed that the *hbpA* was restricted to serovars belong to *L. interrogans.* This observation when extended to clinical isolates obtained from different geographical locations was re-affirmed when 51 of 91 clinical isolates that were PCR positive, were found to belong to *L. interrogans*.

The iron-regulated expression of virulence determinants is well studied in several bacterial systems. In this study, we studied the influence of iron levels on the expression of leptospiral sphingomyelinases. We first demonstrated that leptospiral sphingomyelinase is secreted into the medium as outer membrane vesicles (OMVs). It was detected as an OMV-associated 42 kDa protein in iron-limited culture of *L. interrogans* serovar Lai and was absent in the corresponding non-pathogenic *L. biflexa*. Interestingly, HbpA was also identified in the OMVs of low-iron grown serovar Lai. The presence of sphingomyelinase, HbpA and LipL32 (hemolysis associated protein, hap-1) led us to hypothesize a concerted action of these three pathogen-specific proteins in the effective lysis of host cells. We identified a 63 kDa protein that was later shown to be TolC. This protein, referred to as TolC[63] showed similar protein folding to *E. coli* TolC, as shown by homology modeling with Insight II modeler. The possible role of the protein as a possible transporter of sphingomyelinases is discussed.

CHAPTER 1

REVIEW OF LITERATURE

1.1. Introduction

Leptospirosis is ubiquitous in distribution and has the dubious distinction of being both an occupational hazard and an anthropozoonosis. In the past decade, leptospirosis has emerged as a globally important infectious disease of considerable public concern and is caused by the pathogenic strains of spirochetal bacteria belonging to the genus *Leptospira* (Waitkins, 1987). It prevails in urban environments of industrialised and developing countries, as well as in rural regions worldwide, but is more common in the tropics where conditions for its transmission are particularly favourable (Bharti *et al*., 2003). Humans are accidental hosts whereas wild, domestic and peridomestic animals serve as reservoir hosts. Infection to humans usually results from direct or indirect exposure to the environment contaminated with urine of leptospiruric animals (Levett, 2001). Infection is facilitated by penetration of leptospires through cuts in skin / mucosa / conjunctiva of eye (Schmid *et al*., 1986).

Leptospirosis shows protean clinical manifestations and can mimic those of viral haemorrhagic fevers. Its clinical presentations in humans are extremely wide, ranging from sub-clinical, mild febrile illness to severe syndrome of multi-organ failure with high mortality that is commonly known as Weil's disease. The severe form includes hepato-renal failure, severe pulmonary haemorrhage with respiratory distress and meningitis (Bharti *et al*., 2003). Most countries in the Southeast Asia region are endemic to leptospirosis. Leptospirosis is grossly under-reported due to lack of simple, rapid and efficient tests for early diagnosis and also vaccines that can elicit protective immunity.

1.2. Historical perspectives of leptospirosis

The icteric form of leptospirosis characterised by renal failure, splenomegaly and jaundice was first reported over 100 years ago by Adolf Weil in Heidelberg (Weil, 1886). It is commonly referred as Weil's syndrome and has become synonymous with leptospirosis. Stimson (1907) demonstrated the presence of spirochetes in renal tubule specimens stained with Levadeti technique from a patient, who was diagnosed to have died of yellow fever and called them as *Spirocheta interrogans,* as the hook at the ends resembles a question mark. Unfortunately, this sentinel observation was overlooked for many years. Later the saprophytic organisms present in fresh water were described and named as *Spirocheta biflexa* (Walbach &

Binger, 1914). In Japan, Inada and Ido (1915) successfully detected leptospires and their specific antibodies in the blood of Japanese miners with infectious jaundice.

The importance of occupation as a risk factor was recognized in early 19th century. The role of the rat as a source of human infection was discovered in 1917 (Ido *et al*., 1917) and while leptospiral disease in dogs was recognized, it took several years to clearly distinguish canine infection due to *L. interrogans* serovars Icterohaemorrhagiae or Canicola. Leptospirosis in livestock was recognized some years later (Alston & Broom, 1958). It has been suggested that *L. interrogans* serovar Icterohaemorrhagiae was introduced to Western Europe in the 18th century by westward extension of the range of *Rattus norvegicus* from Eurasia (Alston & Broom, 1958). Several monographs provide extensive information on the early development of knowledge on leptospirosis (Van Thiel, 1948; Wolff, 1954; Alston & Broom, 1958; Faine, 1994; Faine *et al*., 1999).

Soon after the breakthrough that Weil's disease was caused by leptospires, several other disease entities were being recognized to have a leptospiral etiology. These include '*nanukayami'* or the Japanese seven-day fever, '*akiyami'* the harvest fever, cane cutter's disease in Australia, rice field leptospirosis in Indonesia, Fort Bragg fever in USA and more recently Andaman fever or Andaman haemorrhagic fever (AHF) in Andaman and Nicobar Islands, India. Much of the basic current knowledge about leptospires and leptospirosis was understood within a decade following the discovery of leptospires and several types of them were recognized during this period (Kmety & Dicken, 1988 & 1993). Till date, several groups identified leptospires and illustrated the zoonotic nature of the disease. Rats were identified as vectors and subsequently several wild and domestic animals were identified as carriers (Faine, 1994).

1.3. Taxonomy and Classification of leptospires

Leptospires are spirochetes, a group of bacteria that diverged early in the bacterial evolution (Paster *et al*., 1991). Leptospires belong to the Division - Gracillicutes, Class - Scotobacteria, Order - Spirochaetales and Family – Leptospiraceae. Leptospiraceae has three genera *viz*., *Leptospira, Leptonema* and *Turneria*. The strain Ictero No.1 of serovar Icterohaemorrhagiae (*Spirochaeta Icterohaemorrhagiae japonica*) was the first isolate of *Leptospira*, which was recovered by Inada and Ido (1915) from a patient suffering from Weil's disease. Since

then about 268 pathogenic serovars and over 60 non-pathogenic, saprophytic serovars have been identified (Johnson & Faine, 1984). Serovar Sichvan (serogroup Sichvan), serovar Hurstbridge (serogroup Hurstbridge) and serovar Portblairi (serogroup Sehgali) are the recent entities (Brenner *et al*., 1999; Vijayachari *et al*., 2004). Additional serovars have been isolated, but have yet to be validly published.

The classification and nomenclature of *Leptospira* is complex. Presently two different classification systems are being used; one is based on serological characterization and other on the genetic relatedness.

1.3.1. Serological classification

Prior to 1989, the serological classification was in use and was based on antigenic relatedness. According to this classification, the genus *Leptospira* was divided into two species namely, the *Leptospira interrogans* comprising all pathogenic strains and the *Leptospira biflexa* containing non-pathogenic, saprophytic strains isolated from the environment (Faine & Stallman, 1982; Johnson & Faine, 1984). *Leptospira biflexa* can be differentiated from *L. interrogans* by the growth of the former at 13°C and growth in the presence of 8-azaguanine (225 mg / mL) and by its failure to form spherical cells in 1M NaCl.

Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen (Johnson & Faine, 1984; Kmety & Dicken, 1993). If more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing, two strains are said to belong to different serovars (International Committee on Systematic Bacteriology, Sub-committee on the Taxonomy of *Leptospira*, 1987). Serovars that are antigenically related have traditionally been grouped into serogroups (Kmety & Dicken, 1993); though serogroups have no taxonomic standing, they have proved useful in epidemiological situation / settings.

1.3.2. Genotypic classification

Genetic heterogeneity among leptospiral serovars was demonstrated by Brendle *et al*. (1974) and DNA hybridization studies led to the definition of 10 genomospecies of *Leptospira* (Yasuda *et al*., 1987). Genomospecies classification includes leptospiral serovars whose DNA show 70% or more homology at the optimal re-association temperature of 55ºC or 60% or more homology at a stringent re-

association temperature of 70ºC and in which the related DNA contain 5% or less unpaired bases. After an extensive study of several hundred strains, workers at the Centers for Disease Control (CDC) more recently defined 16 genomospecies of *Leptospira* that include those described previously (*L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. inadai, L. biflexa, L. meyeri, L. wolbachii*) (Ramadass *et al*., 1992; Yasuda *et al*., 1987) and five new genomospecies (Brenner *et al*., 1999) including *L. alexanderi* and *L. fainei*, the latter containing the new serovar, Hurstbridge (Perolat *et al*., 1998). DNA hybridization studies have further confirmed the taxonomic status of the genera *Leptonema* (Brenner *et al*., 1999; Ramadass *et al*., 1990) and *Turneria* (Levett *et al*., 2005), both of them having one species each (*L. illini* and *T. parva*). The genotypic classification of leptospires is supported by multilocus enzyme electrophoresis data, but recent studies suggest that further taxonomic revisions are likely (Letocart *et al*., 1999).

The genomospecies of *Leptospira* do not correspond to the previous two species (*L. interrogans* and *L. biflexa*) and indeed, pathogenic and non-pathogenic serovars occur within the same species. Thus, neither serogroup nor serovar reliably predicts the species of *Leptospira*. Moreover, studies (Brenner *et al*., 1999) have included multiple strains of some serovars and demonstrated genetic heterogeneity within serovars. In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* sensu lato from *L. biflexa* sensu lato do not differentiate the genomospecies (Brenner *et al*., 1999; Yasuda *et al*., 1987). The molecular classification poses a problem for the clinical microbiologist, because it is clearly incompatible with the system of serogroups, which has served clinicians and epidemiologists well for many years. Until simpler DNA-based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic leptospires for the foreseeable future. In addition, the retention of *L. interrogans* and *L. biflexa* as specific names in the genomic classification also allows nomenclatural confusion. The merits of molecular classification include the identification of distinct subtypes, as seen with the serovar Hardjo. Two genotypes of serovar Hardjo, with distinct biological characteristics and geographical distribution, named Hardjoprajitno and Hardjobovis are classified within *L. interrogans* and *L. borgpetersenii* respectively (Thiermann *et al*., 1986; LeFebvre *et al*., 1987; Ellis *et al*., 1988).

1.4. Biology and characteristic features of leptospires

Leptospires are highly motile, obligate aerobic spirochetes that share features of both Gram-positive and Gram-negative bacteria (Haake, 2000). Leptospires are about 0·25 x 6 – 25 µm in size and can pass through 0·45 µM filters. Dark-field or phase-contrast microscopy of wet preparations is required for direct visualisation of leptospires, since the bacteria stain poorly (Fig. 1a). Electron microscopy shows a cylindrical cell body (protoplasmic cylinder) wound helically around an axistyle (0·01 – 0·02 µm in diameter), which is comprised of two axial filaments (a spirochetal form of a modified flagellum) inserted sub-terminally at the extremities of the cell body, with their free ends directed towards the middle of the cell (Hovind-Hougen, 1976). An external sheath envelops the axistyle and protoplasmic cylinder, which is demarcated by a cytoplasmic membrane. The axial filament is attached to the inner surface of the membrane and periodically contracts, causing spiral rotatory movement, thus providing mobility to the organisms (Charon & Goldstein, 2002) (Fig. 1b).

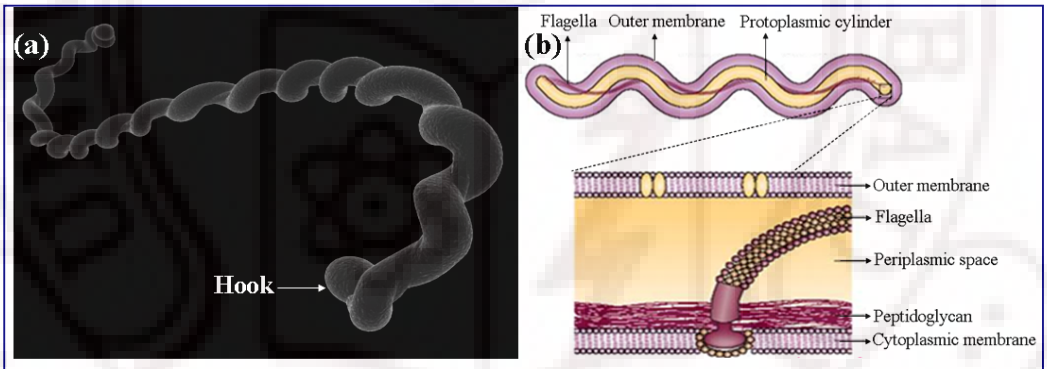


Fig. 1. (a) Scanning electron micrograph of *Leptospira* (reproduced from www.leptospirosis.org) and (b) flagellar and outer membrane organisation of *Leptospira* that shares both Gram-positive and Gram-negative features (reproduced from www.brown.edu).

The appearance and motility of leptospires varies with the nature of the medium in which they are grown. In liquid media, cells appear bent or hooked at one or both ends, although straight mutants do exist. In some cultures, leptospires may appear as small granules (1·5 – 2·0 µm in diameter) containing coiled remnants of the leptospiral cell. Three types of movements are possible such as rotation around a central axis, progressive movement in the direction of the straight end and circular motion. In semi-solid media, motion is by means of flexion. Newly isolated

leptospires appear shorter on initial sub-culture with even higher translational and helical motility (Ellis *et al*., 1983).

Leptospires are fastidious and can not be cultivated in simplified media. These organisms require vitamin B1 and B12 for optimal growth and in some labs it is common practice to use 10% rabbit serum that is rich in vitamin B12 (Ellis & Michno, 1976). Further, leptospires can not utilize carbohydrates as carbon and energy source. Several media are reported for the successful cultivation of *Leptospira in vitro*. A commonly used medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Ellinghausen & McCullough, 1965 (a); Ellinghausen & McCullough, 1965 (b); Johnson & Rogers, 1967), which contains 1% bovine serum albumin and Tween 80; the long-chain fatty acids in the latter serve as the carbon and energy source and available as commercial formulations. Serum-containing liquid or semi-solid media include Korthof's (peptone, NaCl, $NaHCO_3$, KCl, $CaCl_2$, KH_2PO_4, Na_2HPO_4) and Fletcher's (peptone, beef extract, NaCl, and agar) (Faine *et al*., 1999). Optimum growth temperature is between 28ºC and 30ºC. Leptospires are catalase and oxidase positive. Cultures should be checked for the presence of contaminating bacteria after 3 – 4 days and sub-cultured after 7 – 21 days, although leptospires can survive in undisturbed liquid culture for months, sometimes years (Faine *et al*., 1999). Media can be made selective by the addition of several antibiotics, the most common being 5-fluorouracil and neomycin sulphate, although polymyxin B, rifampicin, and vancomycin have been used (Ellis & Michno, 1976). Addition of these antibiotics is done for cultivating these organisms from clinical specimens.

1.4.1. Leptospiral membrane architecture

Leptospira exhibits a surface architecture that resembles Gram-negative and Gram-positive bacteria. Double membrane constitution separated by a periplasmic space supports Gram-negative bacteria whereas attachment of peptidoglycan to the inner membrane resembles Gram-positive nature (Holt, 1978) (Fig. 1b & 2). Thus this bacterium is susceptible to the antibiotics which are used for both Gram-negative as well as Gram-positive bacteria. The bacteria are sensitive to wide range of antibiotics except chloromphenical as some of the serovars were found to be resistant. Several techniques have been developed for the isolation of leptospiral OMPs, which include SDS treatment of salt altered cells (Auran *et al*., 1972; Nunes-Edwards *et al*., 1985; Brenot *et al*., 2001), Sarkosyl extraction (Nicholson & Prescott, 1993), Triton X – 114

extraction followed by phase partitioning (Haake *et al*., 1991; Zuerner *et al*., 1991) and isolation of outer membrane vesicles (OMVs) by alkaline plasmolysis followed by sucrose gradient ultracentrifugation (Haake & Matsunaga, 2002). The optimization of conditions for the efficient extraction of the OMPs has shed light on the structure of the OM in the leptospires. For example, the inclusion of 1 M NaCl, 2 mM EDTA and a pH of 9.0 to hypertonic sucrose indicate that salt bridging or hydrogen bonding between charged amino acids play an important role in stabilizing the leptospiral OM. Divalent cations are probably essential for interactions between leptospiral LPS molecules, as addition of EDTA chelates the divalent cations and helps in the release of the OMPs.

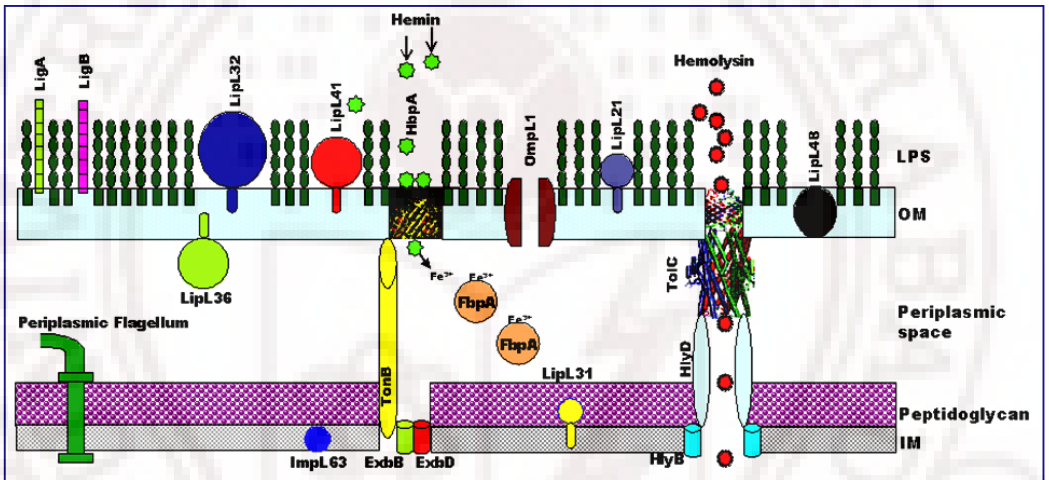


Fig. 2. Model of leptospiral membrane architecture. Leptospires have two membranes, an outer membrane (OM) and a cytoplasmic or inner membrane (IM). As in Gram-positive bacteria, the peptidoglycan cell wall is closely associated with the IM. The leptospiral- surface is dominated by lipopolysaccharide (LPS) carbohydrate side chains. Sub surface proteins include the cytoplasmic proteins and the periplasmic flagellum. The IM contains lipoproteins such as LipL31, ImpL63 and other TonB-related proteins. The OM contains lipoproteins including LipL41 and LipL36, transmembrane proteins including the porin, OmpL1 and novel types of outer membrane proteins (OMPs), including TonB-dependent hemin-binding proteis (HbpA) involved in nutrient acquisition.

Based on the isolation techniques, three classes of leptospiral OMPs have been described as transmembrane, lipoprotein and peripheral membrane proteins (Fig. 2). Haake *et al.* and his group (1991, 1993, 2000 & 2002) have made major contributions to the analysis of the leptospiral membrane proteins. The porin OmpL1, the first leptospiral OMP to be described (Haake *et al*., 1993) is a transmembrane protein present as a trimer. The second class of leptospiral OMPs, the lipoproteins, constitute the most abundant of the leptospiral proteins in the outer membrane, to which they are anchored by fatty acids. The leptospiral lipoproteins include LipL32 (also called as

hemolysis-associated protein-1; Hap-1), LipL36, LipL41, LipL31, LipL21, LipL45 and LipL48. LipL31 is located on the inner membrane whereas LipL21 (Cullen *et al*., 2002 & 2003), LipL32 (Haake *et al*., 2000), LipL41 (Shang *et al*., 1996), LipL36 (Haake *et al*., 1998), LipL45 (Matsunaga *et al*., 2002) and LipL48 are found in the outer membrane. These surface-exposed lipoproteins are conserved among many pathogenic *Leptospira* serovars and are also expressed during infection. LipL45, also called Qlp42 (Nally *et al*., 2001) is produced as a 45 kDa lipoprotein and it is processed to a 31-kDa C-terminal form, $P31_{LipL45}$ (Matsunaga *et al*., 2002). Prediction about the location of these lipoproteins has been facilitated by the presence of charged amino acid residues within their sequence. In LipL31, there is a Glu residue at +3 position, while in LipL36 and LipL48 there is a positively charged residue in the +2 position after the first residue Cys at +1. LipL32 and LipL41 have neutral amino acids in both +2 and +3 positions. Among the third class of proteins, classified as peripheral membrane proteins, the protein $P31_{LipL45}$ can be released from membranes by urea and can be partitioned into both Triton X – 114 detergent and aqueous phases (Haake *et al*., 2000 & 2002).

1.5. Epidemiology

Leptospirosis is one of the most wide-spread zoonosis in the world, reflecting the ability of the causative spirochetes to adapt to the renal tubules of a wide variety of mammalian reservoir hosts (Levett, 2001). Transmission to humans usually occur either by direct or indirect contact with the urine of an infected animal. The incidence is significantly higher in tropical countries than in temperate regions (Ratnam, 1994), which is mainly due to longer survival of leptospires in warm and humid environment. However, in most tropical developing countries, there are greater opportunities for exposure of the human population to infected animals that include livestock, domestic pets, wild or feral animals. The disease is wide spread resulting in outbreaks during rainy seasons.

1.5.1. Leptospirosis outbreaks in India

The isolation of causative organism of leptospirosis was first reported as early as 1931, from the Andaman and Nicobar islands (Taylor & Goyle, 1931). Leptospiral serovars Icterohaemorrhagiae, Pomona and Canicola were reported in suspected cases of PUO (Pyrexia of Unknown Origin) and in patients with jaundice in Delhi in 1966

(Joseph & Kalra, 1966). In 1967, in Bombay, one of the 150 sera from infective hepatitis cases showed evidence of *Leptospira* infection due to *L. pyrogenes* (Bhatnagar *et al*., 1967). Later, human leptospirosis was reported in several states in India, sporadically or as outbreaks, especially during rainy seasons (Ratnam *et al*., 1983; Ratnam, 1994; John, 1996 & 2005; Sehgal, 1998; Sehgal, 2000; Natarajaseenivasan *et al*., 2002; Bharadwaj *et al*., 2002). Sero-prevalence rate of more than 55% was observed in the general population of North Andamans (Sehgal *et al*., 1994). In a study of random sampling of human population, 54% sero-prevalence rate was observed among healthy population from the North Andaman, Andaman & Nicobar archipelago (Murhekar *et al*., 1998). Studies from South India include reports of leptospirosis in several places in states of Tamil Nadu, Kerala and Karnataka (John, 1996; Natarajaseenivasan *et al*., 2002; Ratnam, 1994).

In the state of Tamil Nadu, consequent to an outbreak of bovine leptospirosis in Chennai, serological evidence of leptospirosis was noticed among human subjects (Ratnam *et al*., 1983). During 1984 to 1985, acute renal failure due to leptospirosis in 19 human patients was reported in Madras (Muthusethupathi & Shivakumar, 1987). In 1988, during the peak monsoon season, serum and urine samples from 40 patients, with a history of fever, vomiting, jaundice, abdominal pain and renal failure, from various hospitals in Madras city revealed that 33 patients (82.5%) had specific leptospiral antibodies as evident by MAT (Venkataraman *et al*., 1991). Outbreaks of leptospirosis are increasingly reported since 1990. Among 54 patients admitted to the Government General Hospital, Madras, during November and December 1990 to 1991 with clinical features of fever, jaundice, myalgia, acute renal failure and conjunctival suffusion, leptospiral antibodies were identified in 2 cases and *L. interrogans* serogroup Autumnalis was isolated from one patient (Muthusethupathi *et al*., 1995). In 1994, an increase in the number of individuals with uveitis was noted at Aravind Eye hospital, Madurai after an epidemic of leptospirosis following severe flooding of the Tamil Nadu in the autumn of 1993; out of 46 patients, 80% of them were positive for leptospiral DNA and 72% were positive by serological tests (Kathryn *et al*., 1998). These studies clearly showed that leptospirosis is a significant health problem in Tamil Nadu that is grossly under-estimated.

In 2000 and 2005, following super cyclone and flood, outbreaks were reported in Mumbai (Sehgal, 2006). The outbreak during the 2005 flooding in Mumbai clearly demonstrates the need for a proper surveillance and control measures during such

times of need. Around 310 cases of leptospirosis, with 27 deaths were reported, giving an incidence of 7.85 per 0.1 million population and a case fatality rate of 8.7%. In contrast, during the corresponding period the year before when no flooding had occurred, the incidence of leptospirosis and case fatality rates were 2.1 per 0.1 million and 7.3%, respectively (Kshirsagar *et al*., 2006). In Orissa, following the super-cyclone that hit the coastal villages, nearly 14% of the studied subjects had febrile illness and serological evidence of leptospiral infection (Faine, 1994).

In the state of Andhra Pradesh, there is no systematic study on human leptospirosis and the disease remains largely under-reported. In a preliminary study on prevalence of leptospirosis among suspected cases of Tirupati region of Andhra Pradesh, *L. interrogans* serovar Hardjo emerged as the predominant serovar in numerous dairy farms (Sharma *et al*., 2006). Velineni *et al*. (2007) reported a retrospective hospital-based study on human leptospirosis in Hyderabad region. In this study, among 55 human sera tested by MAT, IgM ELISA and LeptoTek Dri-dot, *L. interrogans* serovar Lai (68%) emerged as a predominant serovar followed by Australis (22%), Autumnalis (8%) and Javanica (2%).

1.5.2. Leptospirosis outbreaks in rest of the world

During the past several years, large outbreaks of leptospirosis have occurred in many parts of the world, particularly in Southeast Asian countries like Philippines and Thailand, in Central and South America (reviewed by Sehgal *et al*., 1995; Sehgal, 2006). The annual incidence of leptospirosis has increased from 0.3 / 100,000 persons (between 1982 and 1995) to 3.3 / 100,000 persons (between 1997 and 1998) in Thailand (Faine, 1994). Besides, leptospirosis was identified as the cause of a significant proportion of cases of non-hepatitis A and E jaundice, non-malarial febrile illnesses and non-dengue haemorrhagic fever in Southeast Asian region (Laras *et al*., 2002). Outbreaks have occurred in Korea on several occasions when the fields were flooded before harvest (Park *et al*., 1989). Outbreaks were reported among general population in Nicaragua, who were exposed to floodwaters (Zaki & Sheih, 1996); acute febrile illness with pulmonary haemorrhage was noted among these patients. High sero-prevalence has also been noticed in some sub-tropical and temperate regions. Sero-epidemiological studies from North-eastern Alpine regions of Italy detected 10% – 12% sero-prevalence of leptospirosis among farmers and forestry workers (Faine, 1994).

El Salvador and Brazil witnessed large outbreaks (Ko *et al*., 1999). During an eight-month period in 1996, the surveillance system detected 326 cases of leptospirosis among the two million population of El Salvador and the case fatality rate was 15%. About 42% of the cases detected by the surveillance system were initially misdiagnosed as dengue fever at the outpatient clinic. Another outbreak was reported in the same year in Rio de Janeiro of Brazil following heavy rainfall (Barcellos & Sabroza, 2001). A random sample of 1067 persons in Seychelles showed a sero-prevalence rate of 37% (Bovet *et al*., 1999). Studies from Yucatan State, Mexico situated in the inter-tropical belt, reported 14.25% (57 / 400) sero-positvity from randomly selected subjects (Vado-solis *et al*., 2002).

1.6. Modes of transmission

The animal hosts play an important role in the transmission of leptospirosis (Levett, 2001). Wild, domestic and peri-domestic animals, like rodents, bats and squirrels serve as reservoir hosts and shed the leptospires in the urine, thereby contaminating the environment. Rodents play a significant role in transmission. Transmission can be direct or indirect. Direct transmission occurs when leptospires from tissues, body fluids or urine of acutely infected or asymptomatic carrier animals enter a new host and establish infection. Presence of leptospires in genital tracts as well as transplacental transmission has been demonstrated in animals (Ellis *et al*., 1978, 1985 & 1986). Indirect transmission occurs when an animal or human being acquires leptospirosis from environmental leptospires, originating in the urine of carrier animals. Leptospires can survive for long periods of time in the environment and probably multiply when the conditions are favourable. The most common portal of entry into new host usually occurs when the pathogen gains entry via the skin through small abrasions or other breaches of the surface integument. They may also enter directly into the bloodstream or lymphatic system via the conjunctiva, the genital tract, the nasopharyngeal mucosa, and the lungs following inhalation of aerosols (Faine, 1994).

Humans are accidental hosts and acquire infection due to several occupational and recreational hazards associated with leptospirosis. Butchers, veterinarians, farmers and rodent control workers are at increased risk (Bolin & Koellner, 1988; Demers *et al*., 1985; Tangkanakul *et al*., 2000). Outbreaks associated with recreational exposure to water have been reported from several countries (Anderson *et*

al., 1978; Sejvar *et al*., 2003). Leptospirosis has been recognized as a potential hazard of water sports, swimming in the river and other recreational activities that expose people to possible contaminated waters. Direct transmission between humans is not indicated. They are not proven to be important epidemiological source of transmission although the excretion of leptospires in human urine occurs for months after recovery has been recorded (Bal *et al*., 1994; Bharti *et al*., 2003). It is thought that the low pH of human urine limits survival of leptospires after excretion. Transmission by sexual intercourse during convalescence has been reported (Harrison & Fitzgerald, 1988).

The transmission cycle of leptospirosis involves the maintenance hosts, the carrier hosts, the environment and human beings (Waitkins, 1987). Almost every known species of rodent, marsupial and mammal can be carrier and excretor of leptospires (Faine, 1994). Maintenance hosts are animals that carry leptospires in their renal tubules, where the bacteria multiply and are shed in the urine for periods varying from months to more than a year (Ellis, 1999; Hathaway, 1985). These animals are essential as sources of infection for other animals or humans. It has been reported that the infecting serovar may be of lower pathogenicity and may cause chronic rather than acute disease in maintenance hosts compared with accidental hosts. For example, pigs are the maintenance hosts for *L. interrogans* serovar Bratislava, *L. interrogans* serovar Pomona and *L. borgpetersenii* serovar Tarassovi. In chronic infections, these leptospiral serovars cause reproductive disturbances in the pigs. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroups Icterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serogroup Ballum. Domestic animals serving as maintenance hosts include dairy cattle for serovars Hardjo, Pomona, and Grippotyphosa, serovars Pomona, Tarassovi, or Bratislava in pigs, Hardjo and Pomona in sheep and Canicola in dogs (Bolin, 2000). Also a single species may carry different serovars in geographically distinct populations, as exemplified by the small Indian mongoose (*Herpestes auropunctatus*), which maintains serovars Sejroe and Icterohaemorrhagiae in Hawaii (Tomich, 1979), serovars Icterohaemorrhagiae and Djatzi in Puerto Rico (Alexander *et al*., 1963), serovars Icterohaemorrhagiae and Jules in Jamaica (Sulzer, 1975), serovars Icterohaemorrhagiae and Brasiliensis in Grenada (Everard *et al*., 1980), and serovar Canicola in Trinidad (Everard *et al*., 1976). Distinct variations in maintenance hosts and the serovars they carry are observed throughout the world. Knowledge of the prevalent serovars and their maintenance

hosts is essential to understanding the epidemiology of the disease in any region (Levett, 2001). The extent to which infection is transmitted depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts.

1.7. Clinical manifestations of leptospirosis

Leptospirosis has protean manifestations that mimic other diseases like dengue, malaria and flu, thus making clinical diagnosis difficult. Pyrexia of unknown origin (Wood *et al*., 2004) includes leptospirosis and necessitates timely lab diagnosis of the disease. Leptospirosis occurs as anicteric leptospirosis in 85% to 90% of the cases, with the severe form or Weil's disease in about 5 - 15% of cases. The clinical presentation of leptospirosis is biphasic, with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Levett, 2001). In anicteric patients, who show flu-like symptoms, the disease is usually self-limiting and they recover without any complications. In Weil's disease, there is involvement of multiple organ systems (Vinetz, 2001) and the clinical presentation depends upon the predominant organs involved. Liver, kidneys and lungs are usually involved and the presenting symptoms may include jaundice, pulmonary haemorrhage, nephritis and splenomegaly.

In 1988 for the first time in India, post-monsoon outbreaks of febrile illness with haemorrhagic manifestations and high case-fatality rates were reported in Andaman Islands. The Andaman haemorrhagic fever (AHF) was attributed to leptospirosis in 1993 (Sehgal *et al*., 1995). In the 1997 outbreaks, pulmonary involvement has been the predominant complication, with haemoptysis as the common symptom. Pulmonary haemorrhage, associated with leptospiral infection have been reported from different parts of the world; serovars implicated include serovar Lai of serogroup Icterohaemorrhagiae in China and Korea (Oh *et al*., 1991), serovar Australis in Australia (Simpson *et al*., 1998), serovar Canicola of serogroup Canicola and serovar Pomona of serogroup Pomona in the 1995 outbreak in Nicaragua (Zuerner & Bolin, 1997; Trevejo *et al*., 1998), serovar Canicola of serogroup Canicola in Orissa, India (Sehgal *et al*., 2002) and serovar Valbuzzi of serogroup Grippotyphosa in Andaman and Nicobar Islands, India (Vijayachari *et al*., 2003). Pulmonary haemorrhage with hepato-renal dysfunction is also common in severe cases. Most patients with renal failure also have significant hepatic

involvement. They show prolonged oliguria, impairment of the excretory functions and uraemic symptoms. Fifty cases of hepato-renal dysfunction of unknown etiology were studied over a two-year period in and around Pune, Maharashtra with evidence of leptospiral infection; 88.2% of the cases were confirmed positive by microscopy and 94% by serology (Sharma *et al*., 2000).

Ocular involvement is also seen as a manifestation of leptospirosis, it is seen both in the leptospiraemic phase as well as in the immunological phase. Of the individual ocular signs, the combination of acute, non-granulomatous pan uveitis, hypopyon (accumulation of puss in the anterior chamber of the eye), vasculitis, optic disc edema, membranous vitreous opacities and absence of choroiditis or retinitis have high predictive value for the clinical diagnosis of leptospiral uveitis (Rathinam, 2005).

In animals, the disease is usually chronic and the animal tends to excrete / shed large numbers of the live organisms in the urine. The disease can result in abortion, haemorrhage (red water urine) and infertility. Using PCR and nucleotide sequence analysis, the presence of *L. kirschneri* was detected in the tissues of the prematurely born foal (Vemulapalli *et al*., 2005). In another study, the serovar Pomona was demonstrated in leptospirosis-associated equine abortions by histological studies and micro-agglutination plate test (Poonacha, 1993). The haemorrhagic syndrome of leptospirosis was studied in guinea pigs. The study correlates hematological, histopathological and immunohistochemical alterations in sixty animals inoculated by the intra-peritoneal route with 1 mL of the culture of virulent strain of *L. interrogans* serovar Copenhageni. Leptospiral antigens were detected by immunoperoxidase staining, chiefly in liver, kidney and heart muscle capillaries. Possible pathogenic mechanisms responsible for haemorrhagic syndrome include toxic and anoxic attacks causing damage to endothelia, platelet depletion and alterations in prothrombin time and fibrinogen concentrations. The clinical laboratory picture is compatible with the histo-pathological observation of disseminated intravascular coagulation in most of the guinea pigs from day 4 of infection (Da Silva *et al*., 1995).

1.8. Immune response of the mammalian host

The clinical presentation of leptospirosis is biphasic (Fig. 3) and thus necessitates the usage of appropriate diagnostic tests. After infection, leptospires

initially appear in the blood circulation and this stage is known as acute or septicemic or leptospiremic phase that last about a week. During this phase, diagnosis can be done using PCR or blood culture. It is followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Edwards & Domm, 1960). In this phase, leptospires are cleared from the body by the host's immune response to the infection. However, they may settle in the convoluted tubules of the kidneys and be shed in the urine for a period of few weeks to several months and occasionally may persist in the eyes for much longer time. The survival of leptospires within the convoluted tubules of the kidneys may be related to the ineffectiveness of the antibody-complement system at this site (Levett, 2001). Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase. During this phase, diagnosis can be done using serological methods or urine culture.

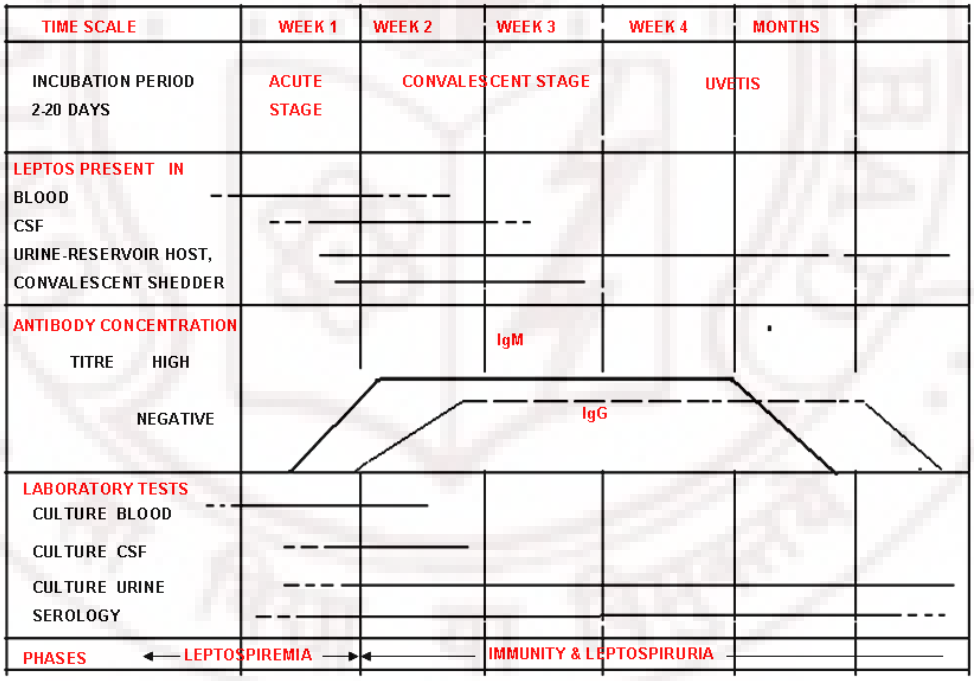


Fig. 3. Biphasic nature of leptospirosis and immune response of mammalian host to the pathogen (reproduced from Levett, 2001)

Immunity to leptospirosis is primarily humoral; cell-mediated immunity does not appear to be important, but may be responsible for some of the late manifestations of the disease. A strong humoral immune response is mounted by the mammalian host (Levett, 2001; Ratnam *et al*., 1984). The antibody response is classical, with peak IgM

levels appearing first, quickly followed by IgG antibodies, which persist longer than IgM (Fig. 3). High IgM levels can be observed during the first two months of the disease. Patients with leptospirosis may produce antibodies that react with several serovars. This phenomenon, called cross-reaction, is often observed in the initial phase of the disease. After the acute phase, cross-reactive antibodies gradually disappear as the immune response "matures", usually in the course of weeks or months, while serogroup- and serovar-specific antibodies often persist for years. Thus the genus-specific antibodies usually remain detectable for months and the serovar-specific antibodies for years.

In leptospirosis, the production of immune complexes leading to inflammation in the central nervous system has been postulated. The levels of circulating immune complexes were correlated with severity of symptoms and their levels fell concurrently with clinical improvement in patients (Galli *et al*., 1985). However, in experimental infections in guinea pigs, leptospires were detected in the kidney interstitium, while immunoglobulin G (IgG) and C3 were deposited in the glomeruli and in the walls of small blood vessels (Yasuda *et al*., 1986). The pathogenesis of equine recurrent uveitis appears to involve the production of antibodies against a leptospiral antigen which cross-react with ocular tissues. Retinal damage in horses with uveitis is related to the presence of B lymphocytes in the retina (Kalsow & Dwyer, 1998). Anti-platelet antibodies have been demonstrated in human leptospirosis that are directed against cryptantigens exposed on damaged platelets and do not play a causal role in the development of thrombocytopenia. Other auto-antibodies that include IgG anti-cardiolipin antibodies and anti-neutrophil cytoplasmic antibodies have been detected in acute illness. However, the significance of anti-neutrophil cytoplasmic antibodies in the pathogenesis of vascular injury in leptospirosis has been questioned. Virulent leptospires induce apoptosis *in vivo* and *in vitro*. In mice, apoptosis of lymphocytes is elicited by LPS via induction of tumor necrosis factor alpha (TNF - α). Elevated levels of inflammatory cytokines such as TNF - α have been reported in patients with leptospirosis (Estavoyer *et al*., 1991).

1.9. Laboratory diagnosis of leptospirosis

The diverse clinical presentations of leptospirosis necessitate definitive laboratory diagnosis. Though culture and direct demonstration of the pathogen is confirmative of the infection, culture from blood and biological specimens is not easy

and is compounded by the difficulty in staining these organisms. Serological methods are the preferred methods of detection due to the strong humoral immunity in this disease (Fig. 4).

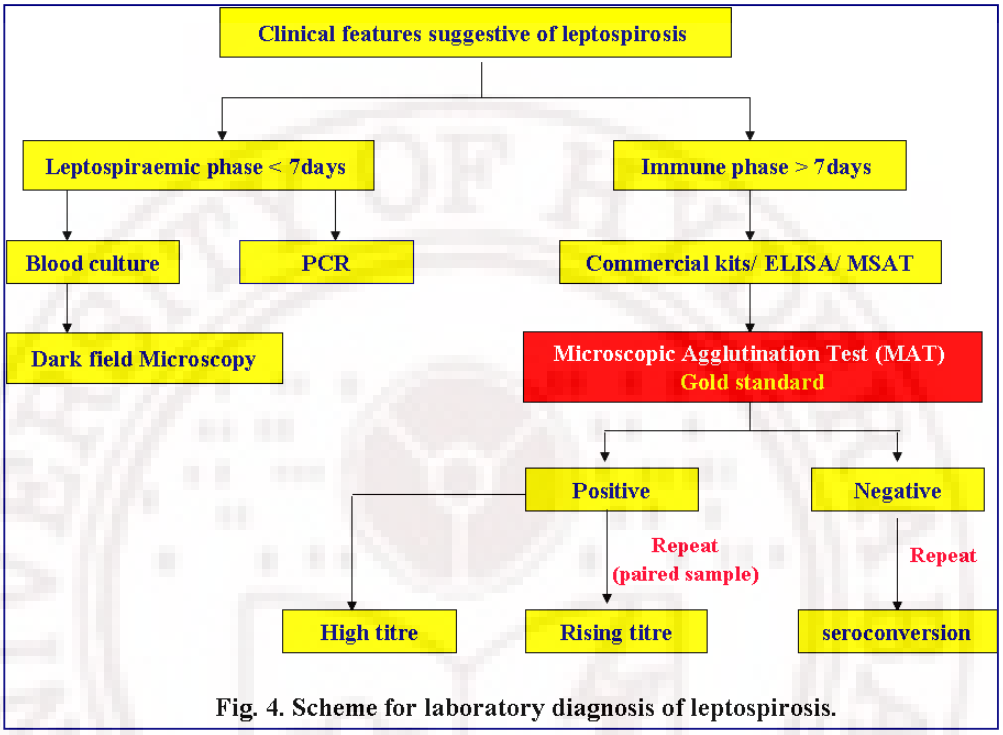


Fig. 4. Scheme for laboratory diagnosis of leptospirosis.

1.9.1. Direct demonstration of leptospires: culture and dark field microscopy (DFM)

The isolation of leptospires by culture depends on the stage of the disease. Leptospires can be isolated from blood and CSF during the leptospiraemic phase, from day 7 - 10 after the onset of fever and initial symptoms, and from urine during leptospiruric phase, from 2nd and 3rd week of illness (Fig. 3) (Levett, 2001). Blood and CSF culture (Wuthiekanun *et al.*, 2007) can be done during leptospiraemic phase and preferably before antibiotics are given. Blood and CSF specimens can be collected in heparin or sodium oxalate for transport at room temperature. Citrate anticoagulation should be avoided since it is inhibitory (Wolff, 1954) and specimens should not be frozen. Media should be inoculated within 24 h. Small inocula consisting of 1 - 3 drop of venous blood are inoculated at the bedside into 5 mL of leptospiral semi-solid media and incubated at 28 - 30°C for several weeks. The main disadvantage of blood culture is that it is difficult, requires several weeks of incubation, has low sensitivity and is not useful during epidemics.

The typical motility of the leptospires in the clinical sample (blood, CSF, urine or peritoneal fluid) observed with dark-field microscopes has been deported; however it is not recommended because of false positivity due to artifacts like lysed RBCs and fibrils that may be mistaken for leptospires. Leptospires in urine may be visualized and / or cultured after the second week of illness in acute diseases and over a prolonged period, up to a year or more in animals, especially in dogs and pigs. Humans do not usually excrete the organisms for more than a few weeks.

1.9.2. Serological methods of diagnosis

Serological tests do not test positive until a few days after infection and the highest titres have been recorded ten days to three weeks after infection (Fennestad & Borg-Petersen, 1962), with antibodies persisting for several months. As many of the areas are endemic, with relatively higher levels of antibodies within a population, it is common to collect a second serum sample after about 3 - 4 days. Sero-conversion with a four-fold rise in titre in paired serum samples in the presence of clinical symptoms is an important criterion for the definitive diagnosis of leptospirosis.

1.9.2.1. Microscopic agglutination test (MAT)

Microscopic agglutination test (MAT) is "Gold standard" for leptospiral diagnosis. It is highly specific and sensitive and is based on the detection of the antibodies against leptospiral lipopolysaccharides (Cumberland *et al*., 1999). Live organisms in the presence of specific antibodies agglutinate and form highly refractive spheroids of various sizes. When maximal degree of agglutination is seen, no free leptospires are visible due to the disintegration of the organisms. The degree of agglutination is usually assessed in terms of the proportion of free leptospires. The accepted endpoint of an agglutination reaction is the final dilution of serum at which 50% or more of the leptospires are agglutinated. As per WHO guidelines, MAT at 1:100 dilution of serum is considered positive. This test is specific for the infecting serovar, although cross-reactivity may be recorded against other serovars within the same serogroup. It is thus necessary to include several serovars, including the prevalent local isolates. A positive diagnosis with MAT can be made with a titre of more than 800 in one or more serum samples (Ko *et al*., 1999).

MAT has been useful in retrospective studies in confirming leptospirosis cases and identifying the prevalent serovar during that period. As mentioned earlier, in the

retrospective hospital-based study done in our lab (Velineni *et al*., 2007), we identified predominant serogroup as Icterohaemorrhagiae. Ismail *et al*. (2006) in a retrospective study on serum samples from patients with undiagnosed acute febrile illness (AFI) and hepatitis cases from Egypt showed that approximately 16% of AFI (141/886) and 16% of acute hepatitis (63/392) cases were positive by MAT with Canicola, Pyrogenes, Pomona, Djasimin, Grippotyphosa and Icterohaemorrhagiae as the causative serovars in AFI, with the latter 3 serovars seen in patients with acute hepatitis. Another long-term study in Portugal and Azores islands showed the utility of MAT in the confirmation of leptospirosis (Vieira *et al*., 2006).

MAT is a commonly used technique for diagnosis of leptospirosis in both domestic and wild animals. The MAT is primarily used as a herd test (Ellis, 1999); usually ten animals, or 10% of the herd, whichever is greater is tested and the vaccination history of the animals documented. As an individual animal test, MAT is useful for diagnosing acute infection: a four-fold rise in antibody titres in paired acute and convalescent serum samples is diagnostic. However, it has limitations in the diagnosis of chronic infection in individual animals and in the diagnosis of endemic infections in herds. In infected animals that shed leptospires in the urine, MAT titres are found to be below 1 / 100, thus limiting its usefulness as a diagnostic test in chronic infections. Boqvist *et al*. (2002), in a study on 424 sow's sera from the Mekong delta in Vietnam, including 283 sows from small-scale family farms and 141 from large-scale state farms showed that the overall seroprevalence was 73 and 29% respectively with titres ≥ 1:100 and ≥1:400. The serovars identified were *L. interrogans* serovar Bratislava in the large scale farms and *L. interrogan*s serovars Icterohaemorrhagiae and Pomona in the small scale farms. Epidemiological study on leptospirosis by Cerri *et al*. (2003), from 1995 to 2001 in Northern and Central Italy included MAT analysis of a total of 9885 serum samples from humans, domestic, and wild animals employing 8 serovars as antigens. Considering sera with ≥1:400 antibody titers as positive, 674 (6.81%) animals scored positive. Sheep, horses, pigs and dogs gave the highest number of positive responses, particularly against the serovar Bratislava and Icterohaemorrhagiae in dogs.

1.9.2.2. Macroscopic slide agglutination test (MSAT)

Macroscopic slide agglutination test (MSAT) is another rapid diagnostic test that is based on agglutination of formalin treated antigens when incubated with

patient's serum. It is less specific when compared to MAT and can not discriminate between antibodies caused by recent infection and residual antibodies of past infection. Sumathi *et al*. (1997) reported that MSAT showed a sensitivity of 97.8% as compared to IgM ELISA and MAT. Brandao *et al*. (1998) reported a 99% sensitivity of MSAT and IgM ELISA when compared to MAT, while Chinari Pradeep *et al*. (1999) that showed 31.4% positivity by MSAT.

1.9.2.3. Enzyme linked immunosorbent assay (ELISA)

Antigens used in ELISA include whole cell sonicate, formalin-extract of a culture of leptospires (Terpstra *et al.,* 1985) and even whole leptospires coated on polysterene microtitre plates (McBride *et al*., 2007). Purified antigens and recombinant antigens have also been used in ELISA. A simplified dot ELISA test with antigen prepared from *L. biflexa* serovar Patoc strain Patoc – 1 (Pappas *et al*., 1985) showed similar sensitivity to multi-antigen MAT. Da Silva *et al*. (1997) evaluated dot ELISA using antigen obtained from *L. interrogans* serovars Brasilinensis, Canicola, Cynopteri, Hebdomadis and Icterohaemorrhagiae and reported that IgM, IgG and IgA antibodies were detected in 98%, 70% and 76% of 63 patients tested. Outer membrane proteins like rLipL32 (Flannery *et al*., 2001; Fernandes *et al.*, 2007), rLipL41 (Flannery *et al*., 2001; Mariya *et al.*, 2006; Senthilkumar *et al*., 2007) and immunoglobulin (Ig)-like Lig proteins (Croda *et al*., 2007; Srimanote *et al*., 2008) were used as antigens in ELISA. Srimanote *et al*. (2008) reported that the diagnostic specificities for the cLigA IgM- and IgG-ELISAs were 98% and 100% respectively.

Several commercial kits are now available in the market for diagnosis of leptospirosis and some of them are evaluated for their efficacy as a screening test for leptospirosis. They include the IgM ELISA kit (PanBio Pvt Ltd., Brisbane, Australia), Serion ELISA kit (Institut Virion / Serion GmbH, Würzburg, Germany), Lepto dipstick (Vijayachari & Sehgal, 2006), LeptoTek Dri-dot (Organon Teknika in collaboration with the Royal Tropical Institute in Amsterdam) (Vijayachari *et al*., 2002; Vijayachari & Sehgal, 2006; Velineni *et al*., 2007), Lepto lateral flow (Vijayachari & Sehgal, 2006) and Latex agglutination test (Organon Teknika). Even though IgM ELISA kit proved to be a useful test during an urban outbreak in Mumbai (Bhardwaj *et al*., 2002), it showed marginally less sensitivity when compared to LeptoTek Dri-dot (Vijayachari *et al*., 2002; Velineni *et al*., 2007). However, most of

these kits need to be imported and are therefore not economically viable for many developing countries.

1.9.3. Molecular methods

PCR has proved to be a useful tool to demonstrate leptospires in tissues and body fluids. Hookey (1992) developed a 16s rDNA – based PCR assay that gave a 631 bp product only in strains from the species *L. interrogans* sensu stricto, *L. borgpetersenii, L. noguchii, L. santarosai, L. weilii, L. inadai, L. meyeri* and the single member strain of *Leptonema*. In contrast, strains representing the saprophytic species *L. biflexa, L. wolbachii* and *L. parva* were not positive. Senthilkumar *et al*. (2001), using a different set of primers amplified a 330 bp product of 16S rRNA gene from clinical isolates. Shukla *et al*. (2003) reported 16s rRNA – based PCR for differentiation of pathogenic and non – pathogenic leptospires. Smythe *et al*. (2002), using real-time PCR of the common region of 16S rDNA was able to differentiate between pathogenic and non-pathogenic species without the need for prior isolation and culture. The method reported by Gravekamp *et al*. (1993) is based on 2 sets of PCR primers, namely G1 / G2 and B64-I / B64-II that amplify products of 285 bp and 563 bp respectively. The former detected *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai and L. meyeri*, while the later identified *L. kirschneri.* Recently, Levett *et al*. (2005) reported *lipl32*-based real-time PCR assay using a 423 bp fragment to detect pathogenic serovars. The test detected all pathogenic serovars, with the exception of *L. fainei* serovar Hurstbridge. Magnetic Immuno PCR Assay (MIPA) developed by Taylor *et al*. (1997) for the rapid detection of leptospires in urine consists of the immuno-magnetic separation of leptospires from inhibitors in frozen formalin-fixed bovine urine prior to PCR detection that resulted in a marked improvement in detection. Kawabata *et al*. (2001) demonstrated that restriction fragment length polymorphism (RFLP) of PCR products of *flaB* gene was an efficient tool for the rapid detection of infected *Leptospira* from clinical specimens. Bolin *et al*. (1989) performed three techniques: nucleic acid hybridization, bacteriological culture and fluorescent antibody test for detection of *L. interrogans* serovar Hardjo type Hardjo-bovis in bovine urine. Ramadass & Marshall (1990) using slot blot hybridization differentiated *L. interrogans* serovar Hardjo strain Hardjoprajitno and Hardjobovis and renamed Hardjobovis as *L. borgpetersenii* serovar Hardjo strain Bovis. Later, Ramadass *et al.* (1997) used RAPD finger printing and showed it to be a

rapid and sensitive method for serovar identification when compared to DNA restriction analysis. Natarajaseenivasan *et al*. (2004) applied this method to screen agricultural workers in Erode, South India in whom the disease was a potential health hazard. The recent *ompL1*- based PCR (Reitstetter, 2006) is specific for *L. interrogans, L. borgpetersenii, L. kirschneri, L. santarosai, L. weilli* and *L. noguchii*.

1.10. Advances in leptospirosis: whole genome sequencing and comparative genomics

The complete genomic sequence of *L. interrogan*s serogroup Icterohaemorrhagiae serovar Lai strain 56601 (Ren *et al*., 2003), *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (Nascimento *et al*., 2004), *L. borgpetersenii* serovar Hardjo strains L550, JB197 (Bulach *et al*., 2006) and *L. biflexa* serovar Patoc strain Patoc 1 (Ames strain) (Picardeau *et al*., 2008) are now available. The genome of serovar Lai was shown to consist of two chromosomes, a 4.33-megabase large chromosome, CI and a 359-kilobase small chromosome, CII, with a total of 4768 predicted genes (Ren *et al*., 2003) (Fig. 5a). Later re-annotation (Bulach *et al*., 2006) of the serovar Lai resulted in the removal of 1206 putative CDS and addition of 52 previously unidentified CDS, making the number of recognized CDS to a total of 3613. Similarly, the genome of serovar Copenhageni was also shown to consist of two chromosomes, a 4.27-megabase large chromosome CI and 350-kilobase small chromosome CII, with a total of 3728 predicted genes (Nascimento *et al*., 2004). Re-annotation (Bulach *et al*., 2006) of the serovar Copenhageni showed that it contains 3530 recognized CDS after removal of 287 CDS and addition of 91 CDS. Both these genomes differ extensively from other related spirochetes, *Treponema pallidum* and *Borrelia burgdorferi* and are much larger than these two spirochetes. Though there was overall genetic similarity between the genomes of serovars Lai and Copenhageni, significant structural differences, including a large chromosomal inversion and extensive variation in the number and distribution of insertion sequence elements were seen. Recently, Bourhy *et al*. (2007) have shown that a large genomic island of size 54 Kb, present in serovar Lai is not seen in serovar Copenhageni. This genomic locus containing 103 predicted coding sequences could excise from the chromosome and form a replicative plasmid, which may have an important role in spreading genes, including virulence factors, among bacterial populations.

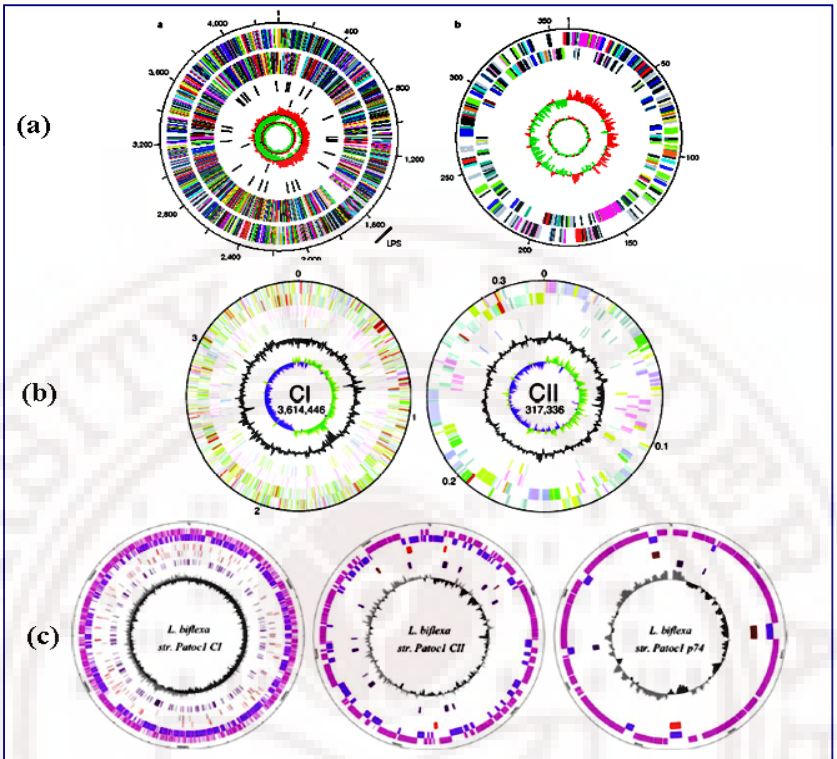


Fig. 5. Circular representation of the genomes of (a) *L. interrogans* strain Lai (Ren *et al.*, 2003), (b) *L. borgpetersenii* serovar Hardjo strain L550 (Bulach *et al.*, 2006) and (c) *L. biflexa* serovar Patoc (Picardeau *et al.*, 2008) with predicted CDSs. Functional information for the large (CI) and small (CII) chromosomes is presented in concentric and genes are colored according to the corresponding functional categories.

The genome of *L. borgpetersenii* serovar Hardjo strain L550 consists of two chromosomes, a 3.61-megabase large chromosome CI and a 317-kilobase small chromosome CII, with a total of 3211 predicted genes (Fig. 5b). The genome of *L. borgpetersenii* serovar Hardjo strain JB197 consists of a 3.57-megabase large chromosome CI and a 299-kilobase small chromosome CII, encoding a total of 3166 predicted genes. *Leptospira borgpetersenii* genome is 16% (approximately 700 Kb) smaller than *L. interrogans* and has a lower coding density indicating of genome decay through a process of insertion sequence-mediated genome reduction. A comparison of these two genomes will yield definitive information, as they are two of the largest phylogenetically distinct pathogenic leptospiral species, which together cause most cases of leptospirosis and encompass 48% of the known serovars (Brenner *et al*., 1999). Although the clinical symptoms of infection due to these two species are

similar, *L. borgpetersenii* does not survive nutrient deprivation and is limited to direct host-to-host transmission cycle, supported by epidemiological data, whereas *L. interrogans*, by virtue of its superior coding capacity, can withstand prolonged nutrient deprivation and maintain a transmission cycle that often involves passage through surface water between mammalian hosts. The gene loss appears to impair tolerance of *L. borgpetersenii* to nutrient deprivation, thereby increasing host dependence relative to *L. interrogans*.

The genome of *L. biflexa* consists of three replicons with a total of 3.96-megabase pairs, with a total of 3590 CDS. The two larger chromosomes are referred to as CI (3.604-megabase) & CII (2.78-megabase) and third circular replicon is the 74.114 kb and is designated as p74 (Fig. 5c). Comparative sequence analysis provides evidence that *L. biflexa* is an excellent model for the study of *Leptospira* evolution as 2052 genes (61%) represent a progenitor genome that existed before divergence of pathogenic and saprophytic *Leptospira* species. Nearly one-third of the genes of *L. biflexa* are absent in pathogenic *Leptospira*.

The *L. biflexa* and *L. borgpetersenii* genomes are similar in size compared to *L. interrogans*, but the gene density (92%) in *L. biflexa* is much higher, compared to *L. interrogans* (75%) and *L. borgpetersenii* (80%), probably as a result of IS mediated genome erosion in *L. borgpetersenii*. Greater gene density as seen in *L. interrogans* can contribute to a relatively stable gene order. It probably an advantage for survival both within mammalian hosts and aquatic environments; *L. biflexa* free-living and can not survive inside a mammalian host while *L. borgpetersenii* is restricted to mammalian host environments only. The presence of large numbers of IS elements is an indicator of genome plasticity in *Leptospira* species. The extensive repertoire of genes encoding proteins involved in signal transduction in *L. biflexa* (287 CDS) compared with *L. interrogans* (214 CDS) and *L. borgpetersenii* (167 CDS) is consistent with an enhanced metabolic capability in *L. biflexa* reflected by its environmental habitat that most probably contributes to its enhanced growth rate relative to the pathogens. Interestingly, genes involved in alginate biosynthesis are present in both *L. biflexa* (11 genes) and *L. interrogans* (8 genes), but are absent in *L. borgpetersenii*, a finding consistent with the reduced environmental survival of *L. borgpetersenii*.

Comparative genomics has been extended to the analysis of outer membrane proteins. There are significant differences in the outer membrane architecture in *L.*

biflexa as observed by the absence of orthologs for LipL32, LipL21, LipL41, LipL36 and LipL45-related proteins, as compared to pathogenic species. As many as 89 of *L. biflexa* lipoprotein genes have no orthologs in other *Leptospira* species, and more than 90 lipoproteins from the pathogenic species have no orthologs in the *L. biflexa* genome. Several putative virulence factors previously identified in pathogenic *Leptospira* spp. are not present in *L. biflexa*, including the Lig surface proteins containing immunoglobulin-like repeats predicted to play a role in the adhesion to host tissues (Choy *et al*., 2007). Similarly, a putative factor H binding protein, LfhA (Verma *et al*., 2006, Barbosa *et al*., 2006) is seen in pathogenic *Leptospira*, but is absent in *L. biflexa*. Although the genome of *L. biflexa* contains putative hemolysins (Louvel *et al*., 2006), its genome is devoid of genes encoding enzymes capable of degrading tissues, such as the range of sphingomyelinases found in pathogenic species (Del Real *et al*., 1989, Segers *et al*., 1990). The role of sphingomyelinases in the pathogenesis of leptospirosis is not clear and it remains to be understood if these sphingomyelinases are key virulence factors or they merely play a role in nutrient acquisition. Their absence in *L. biflexa* strongly supports their involvement in survival within mammalian hosts. Interestingly, the membrane protein, Loa22, the only protein to date that has been shown genetically to be required for virulence in *L. interrogans* (Ristow *et al*., 2007) has a *L. biflexa* ortholog with 73% similarity. Its role in either pathogenic or saprophytic species is unknown, but its presence in the saprophytic species suggests that it is involved in survival rather than being a direct virulence factor and is consistent with the common progenitor hypothesis.

1.11. Prevention and Control

As in any disease control, control measures include timely diagnosis and chemotherapy coupled with prophylactic measures like vaccination. As discussed above, antibody-based methods of detection are preferred as potential diagnostic tools; however, the cost of importing these kits limits their application in clinical laboratories, especially in rural settings. Prevention of leptospirosis can be done by identifying the source and interrupting the transmission (Faine, 1994). However preventive measures to block transmission can be practiced by adhering to maintenance of hygiene and prevention of infection by using protective clothing and footwear.

1.11.1. Chemotherapy

Though several cases of leptospirosis resolve spontaneously, treatment with pencillin and doxycycline is preferred when a definitive diagnosis of leptospirosis is made. Antibiotic treatment is effective within 7 to 10 days of infection and it should be given immediately on diagnosis or suspicion. The antibiotic of choice is benzyl penicillin by injection in doses of 5 million units per day for five days (Watt *et al*., 1988). Doxycycline 100 mg twice daily for 10 days is also recommended. Patients who are hypersensitive to penicillin may be given 250 mg of erythromycin, 4 times daily for 5 days. Tetracyclines are also effective but contraindicated in patients with renal insufficiency, in children and in pregnant women (Bharti *et al.*, 2003). Chemoprophylaxis with doxycycline proved to be very effective in a study by Takafuji *et al*. (1984) on soldiers visiting endemic areas and was found to be almost 100% effective, while in another study (Sehgal, 2000), only 54% protection was seen.

1.11.2. Preventive measures

Although chemoprophylaxis may be feasible for travellers, it is impractical for large high risk populations. In addition, persons who travel in leptospirosis endemic areas should be informed that bathing and swimming in rivers, ponds and other water reservoirs may be hazardous in rat infected areas (Koutis, 2007). Water purification should be implemented as some outbreaks have been associated with drinking of contaminated water. Rodent vector control preferably through the use of slow acting rodenticides and improved hygiene may be some of the measures for diminishing the risk of leptospirosis transmission. Occupational hygiene (in sewers, farmers, and other high risk groups) that includes the use of water proof shoes and gloves is fundamental for preventing human leptospirosis (Koutis, 2007). These occupational groups as well as adventure travellers should also be informed that skin abrasions should not be exposed since they serve as portal of entry for infection. Another control measure that is critical for the disease prevention is the appropriate drainage of wet areas and this is of the most radical means of sanitation. More importantly, policy makers and public health officials should be convinced that addressing the principal conditions of poverty such as poor sanitation could lead to disease elimination. In conclusion, prevention is largely dependent on sanitation measures that are difficult to implement, especially in developing countries.

1.11.3. Leptospiral vaccines

Vaccines for the control of leptospirosis have not made an impact on control measures. Leptospiral lipopolysaccharides (LPS), by virtue of their high immunogenicity were initially considered as ideal vaccine candidates; however, the variation in composition of LPS among the leptospiral serovars afforded low levels of cross-protection, thereby limiting these antigens as vaccine candidates (Faine *et al.,* 1974; Adler & Faine, 1978; Srivastava, 2002).

1.11.3.1. Bacterins

Inactivated whole organisms, referred to as bacterins were used initially as vaccines. They are made by inactivating the leptospires with heat / formalin / ethanol / phenol / irradiation. They have been evaluated in cattle, sheep, horses, swine and dog, as well as in human volunteers (Broom, 1949; Brunner & Meyer, 1950; Brown *et al*., 1955; Chandrasekaran, 1999). The sera from persons vaccinated with a bivalent whole cell inactivated vaccine of *L. interrogans* serovar Hardjo and serovar Pomona contained IgM specific to both serovars (Chapman *et al*., 1990). Although the only bivalent Hardjo / Pomona leptospiral vaccine licensed for humans is being produced in Cuba since 2006, it is used only as veterinary vaccine. In an experimental study on dogs, challenge studies showed high level of protection with a duration of immunity for a year (Klaasen *et al*., 2003). The disadvantages of the inactivated leptospiral vaccines include low-efficacy, short term immunity, with higher doses of vaccination required for protection.

1.11.3.2. Subunit vaccines

Subunit vaccines based on leptospiral OMPs is discussed in detail in section 1.12.4.

1.11.3.3. DNA vaccines

Two leptospiral DNA vaccines have been reported, namely the DNA vaccine encoding LipL32 (Hap-1) that afforded partial protection in gerbils when challenged by pathogenic strains of *Leptospira* (Branger *et al*., 2005) and the endoflagellin gene *flaB2* based DNA vaccine used in guinea pigs (Dai *et al*., 2000). In a recent report, Faisal *et al*. (2008) demonstrated the protective efficacy of LigA DNA vaccine in hamsters, when challenged with *L. interrogans* serovar Pomona.

The current emphasis is to identify protective antigens that will provide long-term protection from a broad range of *Leptospira*. Probably the greatest barrier to anti-leptospiral vaccine development is the practicality of developing a polyvalent leptospirosis vaccine for human beings in endemic areas who may be exposed to several serovars. The outer membrane of leptospires is in contact with the immediate environment of the mammalian host. It can sense and respond to changes effected by the host to counter the invading pathogen. An analysis and identification of novel OMPs in response to conditions encountered *in vivo* will aid in the development of novel vaccine candidates.

1.12. Novel leptospiral proteins as protective immunogens

1.12.1. Leptospiral OMPs expressed *in vivo*

The identification of the subset of outer membrane proteins exposed on the surface of a bacterial cell is critical to understanding the interactions of bacteria with their environments and greatly narrows the search for protective antigens of extracellular pathogens. The leptospiral surfaceome was found to be predominantly made up of a small number of already characterized proteins, being in order of relative abundance on the cell surface: LipL32 > LipL21 > LipL41 (Cullen *et al*., 2005).

LipL36, a prominent OMP of culture derived *L. kirschneri* was not detected in infected hamster kidney tissue by immunohistochemistry, indicating that it is not expressed *in vivo* (Haake *et al*., 1998; Barnett *et al*., 1999). However other proteins like OmpL1, LipL41 as well as LPS were demonstrated in the renal tissue. Though the exact role played by these proteins in host-pathogen interrelationship is not known, they are implicated in renal damage. Haake *et al.* (2000) demonstrated that the LipL32 protein of *L. kirschneri* was found to be a major antigen not only in infected hamsters but also in infected human patients. Recently, Gamberini *et al.* (2005) using immune sera of leptospirosis patients identified 16 proteins as potential vaccine / diagnostic candidates. Out of these sixteen, 4 proteins such as OMPAL 21, LipL 23, LipL32, OMPL31 and MPL36 were not expressed in *L. biflexa* and conserved among pathogenic leptospires.

Proteins expressed during mammalian infection may serve as determinants in leptospiral pathogenesis and as targets for the host immune response. Immunoblot analysis using sera from 105 patients from Brazil and Barbados identified 7 proteins;

p76, p62, p48, p45, p41, p37 and p32, as the targets of the humoral immune response during natural infection (Guerreiro *et al*., 2001). Two-dimensional immunoblots identified four infection-associated antigens, namely LipL32, LipL41 and the two heat shock proteins GroEL and DnaK (Guerreiro *et al*., 2001). Natarajaseenivasan *et al*. (2004), in their observations on leptospiral proteins expressed during acute and convalescent phases of human leptospirosis by using IgM and IgG immunoblots showed the IgG recognition for the leptospiral proteins p32, p41/42, p58, p62 and p82. The IgG response increased considerably against the same proteins during convalescent phase. The response of IgM recognition was same for acute phase as well as convalescent phase sera for the leptospiral proteins p14, p25, p32 and p41/42, respectively. HlyX, a hemolytic protein from pathogenic *Leptospira* was shown to react with patients sera collected during the second week of infection, indicating that the protein is presented to the host immune system during infection (Hauk *et al*., 2005).

LruA and LruB are two novel lipoproteins that are implicated in leptospiral uveitis in horses (Verma *et al*., 2005). A 24 kDa protein identified independently by two groups (Verma *et al.,* 2006; Barbosa *et al.,* 2006)) and referred to as LfhA and Lsa24 is now called as LenA (leptospiral endostatin-like protein A). Subsequently, LenB, LenC, LenD, LenE and LenF have been identified in the genome of *L. interrogans* (Stevenson *et al*., 2007). The apparently widespread distribution of '*len*' genes among virulent leptospires, their presence in multiple copies in *L. interrogans* genome, and their absence from non-pathogenic *Leptospira* species, suggest that Len proteins probably plays an important role in pathogenesis by binding to fibronectin, laminin and regulators of complement activity and provide a selective advantage during mammalian infection. Another surface expressed OMP associated with virulence is Loa22 (Ristow *et al.,* 2007).

1.12.2. Temperature-regulated proteins

The growth of *Leptospira* in standard lab media involves the growth of organisms at 30°C. However, they are subjected to 37°C within the mammalian host. Reports on temperature-regulated proteins include the heat shock proteins such as GroEL, DnaK, Hsp15 and the peripheral membrane protein $P31_{LipL45}$, also known as Qlp42 (Ballard *et al*., 1998; Stamm *et al*., 1991; Nally *et al*., 2001). Temperature-regulated expression of LipL36 has been reported by Cullen *et al.* (2002), who also

demonstrated that LipL32 underwent cleavage to yield products of varying mass and pI at higher temperature. Whole genomic profiling of leptospires maintained at different temperatures (Qin *et al*., 2006; Lo *et al*., 2006) showed up-regulation of several proteins, including the hsp20 heat shock proteins, an outer membrane TolC protein encoded by LA3927 and proteins associated with signal transduction, chemotaxis and motility, membrane biogenesis genes, intracellular trafficking and secretion, information storage and processing.

1.12.3. Lig proteins: osmolarity induced OMPs

The LigA and LigB adhesins in pathogenic *Leptospira* were demonstrated to be induced by osmolarity with enhanced release of LigA into the extracellular environment and increased surface exposure of LigB, as determined by surface immunofluorescence *(*Matsunaga *et al*., 2005). Whole-genome microarray analysis of *L. interrogans* serovar Copenhageni, grown under different osmotic conditions (Matsunaga *et al*., 2007), showed that the transcript levels of 6% of the genes were significantly altered. They were predominantly signal transduction genes. These genes were absent or were pseudogenes in *L. borgpetersenii* serovar Hardjo. It is thus believed that *L. interrogans*, by virtue of the induction of these genes was able to adapt better to diverse environmental conditions.

1.12.4. Recombinant OMPs as potential vaccines

Recombinant protein vaccines have shown considerable vaccine potential. Several OMPs and lipoproteins have been tested as subunit vaccines in experimental animals, with varying success that include leptospiral outer membrane protein OmpL1, lipoprotein LipL41 (Haake *et al*., 1999; Guerriero *et al*., 2001), LipL32 or Hemolysis-associated protein-1 (Hap-1) (Haake *et al*., 2000; Branger *et al*., 2001; Maneewatch *et al*., 2008) and immunoglobulin-like (Lig) protein (Palaniappan *et al*., 2002). Haake *et al*. (1999) for the first time demonstrated the synergistic immunoprotective effects of OmpL1 and LipL41 in Golden Syrian hamster model of leptospirosis; when used alone neither OmpL1 nor LipL41 were effective, but in combination the results were encouraging. LipL32 (Haake *et al*., 2000) and LipL41 (Guerriero *et al*., 2001), by virtue of their ability to induce high levels of antibodies in a natural infection are considered as targets for vaccine design. Several expression systems have been tested, while adenovirus-mediated OmpL1 failed to protect gerbils

against heterologous *Leptospira* infection, adenovirus mediated Hap-1 resulted in significant protection (Branger *et al*., 2001). Recently Seixas *et al.* (2007) showed the potentiating effect of using rBCG as a vector for LipL32. Maneewatch *et al*. (2008) demonstrated the protection offered by monoclonal antibodies of LipL32 in hamster model against lethal infection by heterologous *Leptospira* spp. Other leptospiral proteins that are potential vaccine candidates include LAg42 (Koizumi & Watanabe, 2003), Loa22 (Koizumi & Watanabe, 2003) and Lk73.5 (Artiushin *et al*., 2004). But these proteins are not tested in animal models for vaccine development. Only Lig, LipL41 and Hap - 1 proteins were approved as vaccines against *Leptospira* in animals.

Many recently reported outer membrane proteins (LAg42, Loa22, Lk73.5), lipoproteins (LipL32, LipL45, LipL21 and GLP) and newly discovered virulence factors (Hsp58, FlaA, FlaB, SphH and ChpK) can help to find more suitable vaccine candidates (Wang *et al*., 2007). With the complete genome sequence available for *L. interrogans* serovar Lai (Ren *et al*., 2003), *L. interrogans* serovar Copenhageni (Nascimento *et al*., 2004), *L. borgpetersenii* (Bulach *et al*., 2006) and *L. biflexa* (Picardeau *et al*., 2008) reverse vaccinology may be useful in the identification of potential vaccine candidates.

1.13. Host-pathogen interactions with specific reference to iron acquisition

Iron is the second most abundant metal after aluminium and the fourth most abundant element in the earth's crust. Iron is an important micronutrient in bacterial system except lactobacilli. Iron, by virtue of its wide redox potential is important in biological systems as it catalyses several biochemical reactions and is associated with the transport of reducing equivalents in the electron transport chain. However, its insolubility at biologicl pH makes it unavailable to bacteria, as it exists as insoluble ferric hydroxides and oxyhydroxides. Nature has perhaps made iron highly insoluble, as excess iron is toxic, due to its catalytic role in the Fenton reaction, resulting in the formation of free radicals (Sritharan, 2000). At physiological pH 7.0, the major form of iron is $Fe(OH)_2^+$ (and not Fe $(OH)_3$ as thought earlier) with a solubility of approximately 1.4 X 10^{-9} M (Chipperfield & Ratledge, 2000) that is too low to support the growth of microorganisms (requiring 10^{-7} M iron). Pathogen face additional iron-deprivation as iron is held as protein-bound iron to transferrin,

lactoferrin, heme and haemoglobin within the mammalian host in the extracellular fluids and to ferritin intracellularly (Andrews *et al*., 2003).

The ability of a pathogen to acquire iron in the mammalian host determines the outcome of an infection. In the host- pathogen interaction, the balance between the ability of a mammalian host to withhold iron from invading microorganisms and the ease with which the microorganism can acquire this iron from the host is critical. Iron limitation is an innate immune defense mechanism of the mammalian host (Kochan, 1976).

1.13.1. Bacterial adaptations to iron-limitation

Microorganisms have adapted to conditions of iron-limitation by the elaboration of novel iron acquisition machineries (Griffiths, 1999; Sritharan, 2000). Two well studied mechanisms include siderophore-mediated (Table 1) and the second mechanism elaborated specifically by pathogenic bacteria is the direct removal of the protein-bound iron by specific receptors for the host-iron containing molecules like transferrin, lactoferrin, hemin and haemoglobin as observed in *Neisseria, Moraxella, Pasteurella* spp.

Table 1. Bacterial siderophores and their receptors, the iron-regulated membrane proteins (IRMPs)

Organisms	Siderophores	Iron-regulated membrane proteins	
		Protein	Mol. wt. (kDa)
Escherichia coli	Ferrichrome	FhuA (Coulton *et al*., 1983)	78
	Enterobactin	FepA (McIntosh & Earhart, 1977)	81
	Ferri citrate	FecA (Wagegg & Braun, 1981)	80.5
	Aerobactin	CirA (Curtis *et al*., 1988)	74
Yersinia enterocolitica	Yersiniabactin	FyuA (Rakin *et al*., 1994)	71.4
Pseudomonas aeruginosa	Pyochelin	Ferri-pyochelin receptor (Sokol & Woods, 1983)	14
	Pyoverdin	Ferri-pyoverdin receptor (Meyer *et al*., 1990)	80
Vibrio cholerae	Vibriobactin	ViuA (Butterton *et al*., 1992; Stoebner *et al*., 1992)	74

1.13.2. Siderophore-mediated iron acquisition

Siderophores are low molecular weight (500-1000 Da) Fe^{3+}-specific high affinity molecules with binding affinity constant K_s ranging from 10^{22} to 10^{50}, they can remove iron from the insoluble $Fe(OH)_3$ and from host-iron binding compounds, but not from heme proteins. As the Fe^{3+}-siderophore is greater than 600 Da, uptake of these molecules is a receptor-mediated process. Many of these iron transport receptors are multi-functional and mediate the transport of other molecules that include vitamin B12 and certain colicins.

Siderophores and their receptors, the iron-regulated membrane proteins are extensively studied in *E. coli* (Neilands, 1990, Griffiths & Chart, 1999) in which six siderophore-mediated iron-transport systems has been demonstrated. FhuA, FepA and FecA are well-characterized as Fe^{3+}-siderophore receptors, facilitating the transport of ferrichrome, ferric enterobactin and ferric citrate respectively. The crystal structures of FhuA (Ferguson *et al*., 1998), FepA (Buchanan *et al*., 1999), and FecA (Ferguson *et al*., 2002) show a similar protein folding, consisting of a β – barrel structure, with a N-terminal plug. The β-barrel structure consists of 22 anti-parallel β-strands with short turns that traverse the OM allowing the siderophore- complex to access the periplasm. The extracellular loops of FepA, FecA and FhuA confer the specificity of the respective receptor for its ligand despite the overall similarity in their structure.

1.13.3. Direct uptake of iron from host proteins

Direct acquisition of the protein-bound iron is effected by elaborating specific cell surface receptor proteins for transferrin (Tf), lactoferrin (Lf), heme and haemoglobin (Braun & Killmann, 1999; Schryvers & Stojiljkovic, 1999).

1.13.3.1. Transferrin and lactoferrin receptors

Transferrin is an 80 kDa bilobed monomeric glycoprotein with a Fe^{3+} ion and bicarbonate ion binding site in each lobe. Tf is present in serum (25 – 44 μM), while Lf, a related glycoprotein is present in mucosal secretions (6 – 13 μM) and is also released by leucocytes at the sites of inflammation. Lf is structurally similar to Tf, but differs in that it is capable of holding iron even under acidic conditions (pH < 6.0). These iron-binding glycoproteins effectively reduce the level of free Fe^{3+} in the body.

Table 2 lists the Tf and Lf receptors in different bacteria. The transferrin receptor consists of two proteins, transferrin binding proteins A and B (TbpA & B)

respectively. The *tbpA* and *tbpB* genes are arranged in an operon (Legrain *et al*., 1993) with the intracellular iron concentration regulating the expression via the iron regulator (see section 1.13.5 for details). The invariant presence of Tf receptor genes in clinical isolates of meningococci and gonococci implies their possible role within the mammalian host. The Lf receptor, first identified as LbpA (Schryvers & Morris, 1988), consists of an additional receptor protein LbpB (Bonnah & Schryvers, 1998) that shares homology with TbpB, but differs in that it has two distinct regions rich in negatively charged amino acids. Both TbpB and LbpB are attached to OM via an N-linked terminal lipid anchor. The operonic organization of the *lbp* genes and regulation of the expression by iron parallels that of the transferrin receptor.

Table 2. Bacterial transferrin and lactoferrin receptors

Organism	IRMPs		Size (kDa)	Reference
	Tf	Lf		
Haemophilus influenzae	Tbp1 Tbp2		100 85	Gray-Owen *et al*., 1995
Neisseria meningitidis	Tbp1 Tbp2		100 65 - 90	Legrain *et al*., 1993
Neisseria gonorrhoeae	Tbp1		37	McKenna *et al*., 1988
		LbpA	103	Biswas & Sparling, 1995
Staphylococcus aureus	Tbp		42	Modun *et al*., 1994
Treponema denticola		Lbps	50 & 35	Staggs *et al*., 1994
Treponema palladium		Lbps	45 & 40	Staggs *et al*., 1994
Borrelia burgdorferi	Tbp		28	Carroll *et al*., 1996
Prevotella nigrescens	Tbp		37	Duchesne *et al*., 1999
Actinobacillus pleuropneumoniae	TfbA Tbp		60 110	Strutzberg *et al*., 1995
Moraxella catarrhalis	TbpA TbpB		~ 115 ~ 80	Myers *et al*., 1998

1.13.3.2. Hemin and haemoglobin receptors

Another important source of iron within the mammalian system is heme. Heme is a rich source of iron. It is present largely in haemoglobin, which is a globular protein with a quaternary structure built from two alpha and two beta subunits, to each of which is bound a heme subunit. Iron bound to haemoglobin (Hb) constitutes nearly two-thirds of the total iron in the human body (Bridges & Seligman, 1995) that it is not readily available to pathogens because of its compartmentalization within

erythrocytes. Small amounts of Hb (80 – 800 nM) are found in normal human serum as a result of spontaneous haemolysis, but it is rapidly complexed by haptoglobin, while heme is rapidly complexed as haemopexin (Sassa & Kappas, 1995).

Several bacteria such as *Vibrio* spp. *S. dysenteriae*, enterohaemorrhagic *E. coli* 0157:H7, *Y. enterocolitica*, *Neisseria* spp. *S. marcescens*, *P. aeruginosa*, *H. influenzae* and *Treponema* spp. utilize heme as an iron source (Table 3).

Table 3. Bacterial hemin and haemoglobin receptors

Organism	IRMPs		Size (kDa)	Reference
	Hemin	Hb		
Treponema denticola	Hemin receptor		47	Scott *et al*., 1993
	HbpA		44	Xu *et al*., 2001
	HbpB		42.8	Xu *et al*., 2001
Haemophilus influenzae	Heme-hemopexin-receptor		61	Hanson *et al*., 1992
		Hemoglobin binding protein	120	Jin *et al*., 1996
Vibrio cholerae	HutA		77	Henderson & Payne, 1994
Vibrio vulnificus	HupA		77	Litwin & Byrne, 1998
Vibrio parahaemolyticus	HupA		77	O'Malley *et al*., 1999
	HupO		77	Ahn *et al*., 2005
Vibrio fluvialis	HemR		78	Stojiljkovic & Hantke, 1992
E. coli O157:H7	ChuA		69	Torres & Payne, 1997
Serratia marcescens	HasR		98	Ghigo *et al*., 1997
Neisseria gonnorrhoeae		HpuB	89	Chen *et al*., 1996
	HmBP		97	Lee & Levesque, 1997
		HmbR & HpuB		Stojiljkovic *et al*., 1995; Lewis & Dyer, 1995

1.13.4. Mechanism of iron uptake from Fe^{3+}-siderophore / hemin receptors

The uptake of Fe^{3+} bound to siderophores and heme occurs via specific outer membrane TonB-dependent receptors (Fig. 6). ExbB and ExbD are integral cytoplasmic membrane proteins whereas TonB is periplasmic and anchored to the cytoplasmic membrane by its hydrophobic N-terminal domain. TonB contains a Pro-rich central domain that is thought to form an extended rigid structure that allows the

TonB protein to span the periplasmic space enabling the C-terminal domain to contact TonB-dependent receptors in the outer membrane.

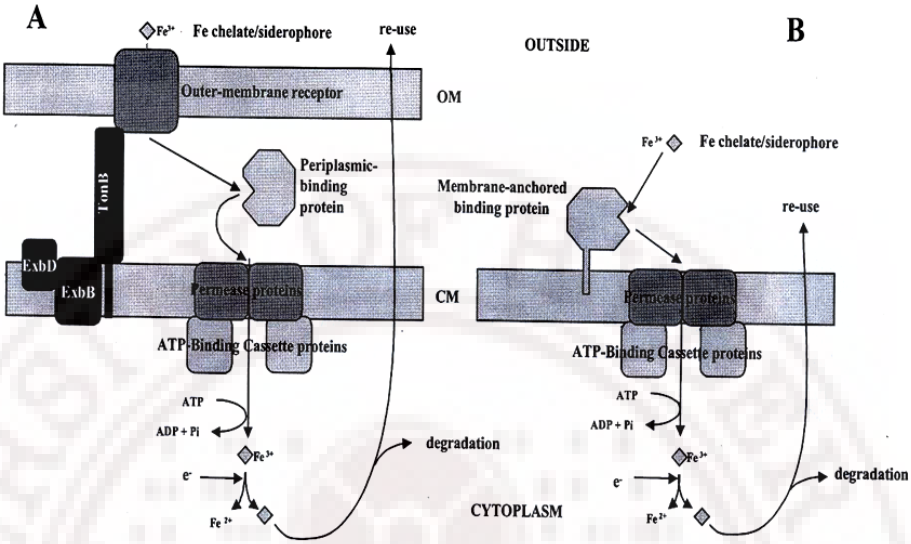
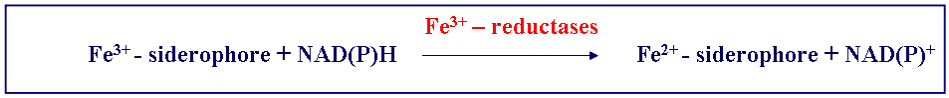


Fig. 6. Diagrammatic representation of iron uptake mechanisms in Gram-negative (A) and Gram-positive (B) bacteria. Reproduced from Andrews *et al.*, 2003.

Fe^{3+}-siderophore and hemin receptors are TonB-dependant outer membrane proteins. Upon binding of the specific ligands, these receptor proteins undergo conformational change and interact with the TonB protein via its TonB box, resulting in opening of the channel of the β-barrel by subtle rearrangements of the loops of the plug domain through which iron / Fe^{3+} - siderophore / hemin molecule is translocated to the inside. This is an active process and requires energy. This energy is provided by the proton motive force of the cytoplasmic membrane and is delivered by the energy-transducing TonB – ExbB - ExbD protein complex working co-ordinately (Braun & Braun, 2002). The TonB protein interacts directly with outer membrane components and act as energy transducer, coupling the cytoplasmic membrane energy to the high-affinity active transport of the Fe^{3+}-siderophores (Fig. 6). The subsequent transport of siderophores into the cytoplasm is facilitated by the periplasmic binding protein (PBT)-dependent transport systems, a subclass of the ABC superfamily of transport proteins. The FhuD, FepB and FecB are the periplasmic binding proteins in *E. coli*, mediating the transport of the respective siderophores. The iron from the Fe^{3+} - siderophore is released as Fe^{2+} by NADH / NADPH -dependant reductases.



The released iron is incorporated into porphyrins or into apo-proteins. The mechanism is similar for hemin receptors. However, the iron may be internalized as hemin-bound iron or it may be released at the cell surface and then internalized.

Another novel mechanism seen in *S. marcescens* involves the secretion of a soluble protein called as hemophore, which chelates the heme from heme proteins and releases the heme at the cell surface, which is internalised via a TonB-dependent receptor (Wandersman & Stojiljkovic, 2000; Ghigo *et al*., 1997). This iron-regulated heme acquisition system encoded by *has* operon consists of the hemophore-specific outer membrane receptor (HasR), the hemophore (HasA) and two specific inner membrane hemophore secretion proteins (HasD and HasE) (Ghigo *et al*., 1997). This hemophore does not directly affect the release of iron from hemoproteins but carries a heme-binding activity and can remove heme from hemoglobin.

1.13.5. Regulation by iron at the molecular level

Intracellular levels controls the expression of the iron acquisition machinery as understood from the exhaustive information in *E. coli* and several bacterial systems. In *E. coli* iron as Fe^{2+} binds to the regulator molecule Fur (Ferric Uptake Regulator encoded by '*fur*' gene) that is a 17 kDa protein and the Fur – Fe^{2+} complex binds to the Fur box / iron box; a 19 bp consensus sequence 5'-GATAATGATAATCATTATC -3' located upstream of the start point of the genes encoding the iron acquisition machinery (Braun *et al*., 1998). When iron is limiting, the repressor molecule, on its own does not bind to the iron box, thereby resulting in the induction of expression of components of the iron acquisition machinery (Fig. 7). In Gram-positive bacteria, the DtxR (Boyd *et al*., 1990), which first identified in *C. diphtheriae* though it is not homologous to Fur, it functions similarly to the latter.

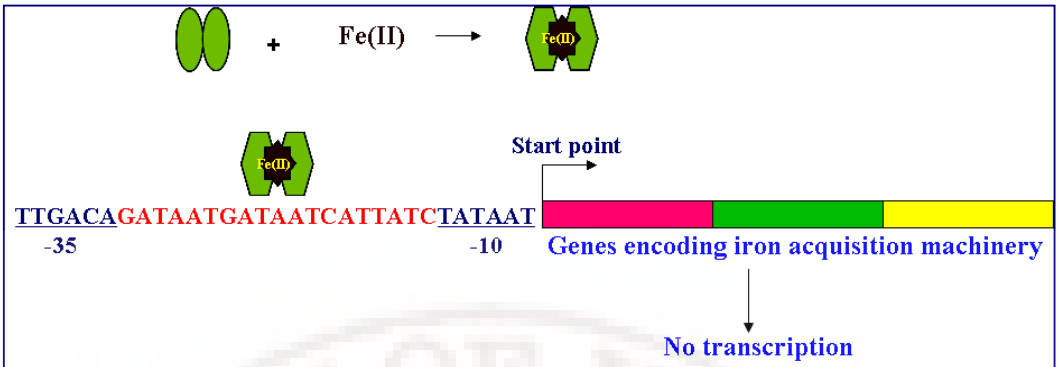


Fig. 7. Schematic representation of regulation by iron at the molecular level.

1.13.6. Iron and bacterial virulence

The intracellular iron levels regulate not only the iron acquisition machinery but also the expression of virulence factors / toxins in several bacterial systems. This was first demonstrated in *C. diphtheriae*. Mode of action is identical to that depicted in Fig. 7 above. Iron has been shown to regulate the Shiga toxin in *Shigella* spp., exotoxin A in *P. aeruginosa*, haemolytic toxin of *V. cholerae,* vero – cytotoxin of enterohaemorrhagic *E. coli* and α-hemolysin in *E. coli* (Litwin & Calderwood, 1993; Sritharan, 2000).

When Fe^{2+} binds to DtxR as a co-repressor molecule, the DtxR - Fe binds to the –10 region upstream of transcription start site of the *tox* gene encoding diphtheria toxin, thus blocking its transcription by RNA polymerase. Thus under low iron conditions, the toxin production is increased (Boyd *et al*., 1990).

Hemolysins are expressed by several bacteria including *V. cholerae* (Stoebner & Payne, 1988), *S. marcescens* (Poole & Braun, 1988), *P. gingivalis* (Chu *et al*., 1991), *A. caviae* (Karunakaran & Devi, 1994) and *E. tarda* (Janda & Abbott, 1993) in whom their expression is linked with iron availability. In the iron-deficient environment of the host, hemolysins act on erythrocytes and other cells, causing cell lysis that release nutrients including iron that can be utilised by the pathogen. The spirochete *T. denticola* produces hemolysins that help to lyse RBC and release the heme from which iron is obtained via a heme-binding protein expressed under iron-deficient conditions (Chu *et al*., 1995).

The relationship between iron and bacterial virulence has been studied in experimental animals. The virulence of the organisms and their multiplication increased significantly upon injection of exogenous iron into these animals, while

reducing the iron availability helped to control the growth of the pathogen and thus the infection. Such animal experiments have been conducted in mice using *S. aureus* (Gladstone & Walton, 1970), *V. cholerae* (Ford & Hayhoe, 1976) and *Y. enterocolitica* (Robins-Brown & Prpic, 1985).

1.14. Iron acquisition in *Leptospira*

Little is known about the mechanism of iron acquisition in *Leptospira* spp., though it is known that iron is an essential nutrient for the growth of *Leptospira* (Faine, 1959). Cullen *et al*. (2002), in their study on outer membrane proteins showed the influence of temperature and iron on the expression of certain outer membrane proteins, namely LipL32, LipL36 and pL50. Fur-like genes are also identified in *Leptospira* genome (Ren *et al*., 2003; Nascimento *et al*., 2004). Genome analysis has also revealed that the *Leptospira* spp. possesses a complete heme biosynthetic pathway and is also capable of using exogenous heme sources (Guegan *et al*., 2003). Louvel *et al*. (2005), using random insertional mutagenesis in *L. biflexa* identified five heme-requiring mutants, three of which had insertions in a gene encoding a protein that shares homology with the TonB-dependent ferric citrate receptor FecA of *E. coli* and the other two mutants showed a *Himar1* insertion into FeoB-like gene, the product of which is required for ferrous iron uptake in many bacterial organisms. However, direct evidence for their role in iron acquisition is not shown. In their recent report, they discuss their observations on iron acquisition in *L. biflexa* in the light of the data obtained from the whole genome sequencing (Louvel *et al*., 2006).

Leptospira do not appear to produce siderophores, and analysis of the *Leptospira* genomes did not allow the identification of any genes that encoded proteins involved in siderophore synthesis or siderophore secretion (Sritharan *et al*., 2005; Asuthkar *et al*., 2007). However they could use exogenous siderophores of other microorganisms as an iron source (Louvel *et al*., 2006). Among the hydroxamate-type siderophores, aerobactin and ferrichrome were used by both *L. biflexa* and *L. interrogans*, while desferrioxamine was only used by *L. biflexa*. The catechol siderophore enterobactin produced by enterobacteria, was not utilized as an iron source by *Leptospira* spp. This *in vitro* utilization of exogenous siderophores suggests that *Leptospira* encounter the corresponding siderophores in their environment. While it is not surprising that *Leptospira* would use exogenous

siderophores as an expedient way to acquire iron, it is not clear why saprophytes have the ability to use hemin and haemoglobin as an iron source.

The first evidence for direct acquisition of iron by a hemin-binding protein in *L. interrogans* serovar Lai was put forth from studies in our lab (Sritharan *et al*., 2005). We identified an 81 kDa iron-regulated outer membrane hemin binding protein (HbpA) using i*n silico* analysis of the genome of *L. interrogans* serovar Lai with ferric enterobactin receptor FepA of *E. coli* and its surface localization and iron-regulated expression was well characterized (Asuthkar *et al*., 2007). The iron-regulated expression of HbpA was further confirmed by the findings of Murray *et al.* (2008) employing real-time RT-PCR analysis and its surface localization was also analysed and supported our findings by Viratyosin *et al.* (2008). Louvel *et al.* (2008) demonstrated that the Hklep/Rrlep regulatory system is critical for the *in vitro* growth of *L. biflexa*, and suggest that this two-component system is involved in a complex mechanism that regulates the heme biosynthetic pathway. Murray *et al.* (2008) identified that *Leptospira* spp. possess a functional heme oxygenase and is required to scavange iron from haemoglobin.

1.14.1. Identification of hemin-binding protein (HbpA) in *L. interrogans*

In silico analysis of the genome of *L. interrogans* serovar Lai with ferric enterobactin receptor FepA of *E. coli* identified a putative TonB-dependent outer membrane receptor (encoded by LB191) (Sritharan *et al*., 2005). In addition to the strong structural similarity with other Fe^{3+}-siderophore receptors, the presence of the fur gene (LB183) and the Fur box in the vicinity of LB191 confirmed that it is an iron-regulated protein and a putative hemin-binding protein and is referred as hemin-binding protein A (HbpA). The full-length HbpA was cloned and the hemin-binding ability of HbpA was shown experimentally by both spectrophtometrically and spectrofluorimetrically (Asuthkar *et al*., 2007). The gene encoding heme oxygenase (LB186) was located in the neighbourhood of *hbpA* and the FRAP-NPNL motif, associated with heme binding is present in HbpA. Despite showing low level of similarity (39%) and identity (22%) with FepA of *E. coli*, the leptospiral homologue revealed features of protein folding like other Fe^{3+}-siderophore receptors (Fig. 8). Homology modelling with Insight II modeller showed that it possessed the characteristic β-barrel structure with the three domains, namely the β-barrel, plug domain and N-terminal TonB box.

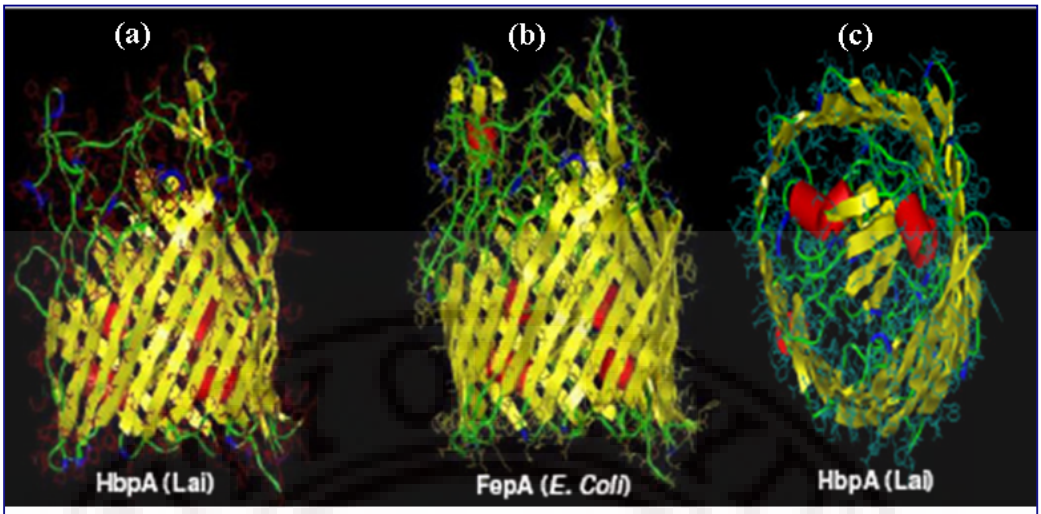


Fig. 8. Homology-modeled structure of hemin-binding protein of *L. interrogans s*erovar Lai. (a) shows the β-barrel structure and (c) - top view showing plug domain. (b) is the FepA of *E. coli*.

1.15. Iron levels and hemolysin expression in *Leptospira* spp.

Genome analysis revealed the presence of several virulence determinants in *Leptospira* spp. (Ren *et al*., 2003; Nascimento *et al*., 2004). Although *Leptospira* virulence factors such as hemolysins (Kasarov, 1970; Thomson & Manktelow, 1986), lipopolysaccharide (Isogai *et al*., 1986), glycolipoprotein (Alves *et al*., 1992)), peptidoglycan (Dobrina *et al*., 1995)), heat shock proteins (Stamm *et al*., 1991), flagellin (Goldstein & Charon, 1990), and others may contribute to the pathogenesis, their pathogenetic mechanisms have not been clearly understood.

Hemolysins are cytolytic toxins found in a wide spectrum of organisms and can be classified as enzymatic, pore forming and surfactant based on the mechanism of action on target cell membranes (Rowe & Welch, 1994). Alexander *et al.* (1956) first reported hemolysins in *Leptospira*. This was followed by several other reports (Alexander *et al*., 1971; Stamm & Charon, 1979; Bernheimer & Bey, 1986; Del Real *et al*., 1989; Segers *et al*., 1990 & 1992; Lee *et al*., 2002). Leptospiral hemolysins are heat-labile and considered to be phospholipases, with phospholipase A and sphingomyelinase A activities demonstrated (Bernheimer & Bey, 1986). Pathogenic *L. interrogans* and non-pathogenic *L. biflexa* both have phospholipase A activity, while the sphingomyelinase C activity was seen only in strains of *L. interrogans* (Kasarov, 1970). Segers *et al*. (1990) cloned and characterized a sphingomyelinase gene (*sphA*) from the serovar Hardjo. Later, the same group based on hybridization

experiments identified putative sphingomyelinase genes (PSGs) in all pathogenic *Leptospira* spp. and demonstrated their absence in the saprophytic members (Segers *et al*., 1992). Lee *et al*. (2002) demonstrated that SphH is a pore-forming hemolysin that lacks both sphingomyelinase and phospholipase activities. Recently, Hauk *et al*. (2005) studied the hemolytic activity of HlyX hemolysin and additionally the potentiating effect of LipL32 on hemolysis.

Studies in our lab (unpublished data) show that iron-deprivation resulted in increased expression of sphingomyelinase, as analysed by RT-PCR. Our present observations on HbpA and the hemolysins are the basis for our objectives in this study.

Objectives of the study

I. **Direct demonstration of HbpA expression upon iron-limitation in *Leptospira*.**
II. **Evaluation of the diagnostic potential of HbpA.**
III. **Iron levels and expression of the sphingomyelinase(s) in *L. interrogans* serovar Lai.**

CHAPTER 2

MATERIALS AND METHODS

2.1. Sources of chemicals

Ellinghausen-McCullough-Johnson-Harris (EMJH) medium base, EMJH enrichment and Noble agar were purchased from Becton and Dickinson, USA. Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethylene diamine tetra-acetic acid (EDTA), ethylene diamine N-N' diacetic acid (EDDA), 2-2' dipyridyl, acrylamide, Ponceau S, hemin-agarose beads, agarose, ethidium bromide, isopropyl β-D-1-thiogalactopyranoside (IPTG), Coomassie Brilliant blue R - 250 and bicinconic acid (BCA) protein estimation kit were purchased from Sigma Aldrich Pvt. Ltd, USA. Nitrocellulose membrane and 0.2 μm syringe filters were purchased from Sartorius, GmbH, Gottingen. 5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium (BCIP – NBT) and anti-IgG alkaline phosphatase (ALP) and fluorescent isothiocyanate (FITC) secondary antibodies were purchased from Bangalore Genei Pvt. Ltd, India. Bacterial grade media components, other analytical reagents and solvents were purchased from Qualigens / Sisco Research Laboratories / Hi media / Bangalore Genei Pvt. Ltd. Bug Buster Ni-NTA His-bind purification kit was purchased from Novagen, USA. DNA gel extraction kit was purchased from Biogene Reagents Inc. CA, USA. Oligo nucleotide primers were synthesized from Imperial BioMedics, France. DNA restriction enzymes, RNaseA, dNTPs, DNA and protein molecular weight markers were purchased from MBI Fermentas, Lithuvania. Microtitre plates were purchased from Corning, USA. Immobilized *E. coli* lysate kit was purchased from Thermo Scientific, USA.

2.2. Sources of bacterial strains and plasmid vectors

The leptospiral serovars used in the study (Table 4) were obtained from the National Repository at the Regional Medical Research Center, ICMR, Port Blair, Andaman and Nicobar Islands, India. Human bacterial pathogens such as *Escherichia coli* strain ATCC25922, *Proteus vulgaris, Staphylococcus aureus* strain ATCC29213, *Pseudomonas aeruginosa strain* ATCC27853 and *Klebsiella pneumoniae* were kindly provided by L.V. Prasad Eye Institute, Hyderabad, India. *Mycobacterium tuberculosis* strain H37Rv and *Escherichia coli* strain DH5α were from our lab collection. *Escherichia coli* strain BL21 (DE3) and pET-28a(+) vector were purchased from Novagen, USA.

2.3. Source of leptospiral DNA

DNA samples corresponding to different outbreak associated clinical isolates (Table 5) were obtained from the collection of Pathogen Evolution Laboratory, Center for DNA Finger printing and Diagnostics (CDFD), Hyderabad, India.

Table 4. List of *Leptospira* used in the study

S.no	Species	Serogroup	Serovar	Strain
I. Pathogenic leptospiral serovars				
1	*Leptospira interrogans*	Icterohaemorrhagiae	Lai	Lai
2	*Leptospira interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
3	*Leptospira interrogans*	Pomona	Pomona	Pomona
4	*Leptospira interrogans*	Australis	Australis	Ballico
5	*Leptospira interrogans*	Autumnalis	Rachmati	Rachmat
6	*Leptospira interrogans*	Hebdomadis	Hebdomadis	Hebdomadis
7	*Leptospira interrogans*	Sejroe	Hardjo	Hardjoprajitno
8	*Leptospira interrogans*	Canicola	Canicola	HU IV
9	*Leptospira interrogans*	Pyrogenes	Pyrogenes	Salinem
10	*Leptospira kirschneri*	Cynopteri	Cynopteri	3522C
11	*Leptospira kirschneri*	Grippotyphosa	Ratnapura	Wumalasena
12	*Leptospira kirschneri*	Grippotyphosa	Grippotyphosa	Moskva V
13	*Leptospira borgpetersenii*	Javanica	Poi	Poi
14	*Leptospira borgpetersenii*	Tarassovi	Tarassovi	Perepelitsin
15	*Leptospira borgpetersenii*	Ballum	Ballum	MUS127
16	*Leptospira santarosai*	Sarmin	Weaveri	CZ390
17	*Leptospira noguchii*	Louisiana	Louisiana	LSU1945
18	*Leptospira noguchii*	Panama	Panama	CZ214K
II. Non-pathogenic leptospiral serovars				
19	*Leptospira meyeri*	Ranarum	Ranarum	ICF
20	*Leptospira biflexa*	Semaranga	Patoc	Patoc - 1
21	*Leptospira biflexa*	Semaranga	Andamana	CH11

Table 5. Leptospiral DNA used for PCR (from the laboratory of Dr. Niyaz Ahmed, CDFD, Hyderabad, India)

S.No	Genome spp.	Serogroup	Serovar	Strain	Region
1	*L. santarosai*	Bataviae	Brasiliensis	An 776	Brazil
2	*L. santarosai*	Sejroe	Guaricura	Bov.G	Brazil
3	*L. santarosai*	-	Lyme	Bovino 131	Brazil
4	*L. meyeri*	-	Ranarum / Semaranga / Patoc	horse	Brazil
5	*L. interrogans*	Canicola	Canicola	M12/90	Brazil
6	*L. santarosai*	Sejroe	Guaricura	M4/98	Brazil
7	*L. interrogans*	Australis	Rushan	L01	Brazil
8	*L. interrogans*	Canicola	Canicola	L02	Brazil
9	*L. interrogans*	Canicola	Canicola	L03	Brazil
10	*L. interrogans*	Canicola	Canicola	L09	Brazil
11	*L. interrogans*	Canicola	Canicola	L14	Brazil
12	*L. santarosai*	Grippotyphosa	Bananal	2ACAP	Brazil
13	*L. interrogans*	Lyme	Lyme	K30B	UK
14	*L. interrogans*	Australis	Australis	K9H	UK
15	*L. santarosai*	Sarmin	Weaveri / Rio	Isolate 2	Costa Rica
16	*L. santarosai*	Tarassovi	Rama	Isolate 3	Costa Rica
17	*L. noguchii*	Pyrogenes	Guaratuba (on DNA info)	Isolate 4	Costa Rica
18	*L. santarosai*	Tarassovi	Rama	Isolate 5	Costa Rica
19	*L. santarosai*	Bataviae	Claytoni	Isolate 6	Costa Rica
20	*L. interrogans*	Unknown	Unknown (Varela?)	Isolate 10	Costa Rica
21	*L. borgpetersenii*	Ballum	Kenya	153	Tanzania
22	*L. borgpetersenii*	Ballum	Kenya	159	Tanzania
23	*L. borgpetersenii*	Ballum	Kenya	723	Tanzania
24	*L. kirschneri*	Icterohaemorrhagiae	Sokoine	745	Tanzania
25	*L. borgpetersenii*	Ballum	Kenya	766	Tanzania
26	*L. kirschneri*	Icterohaemorrhagiae	Sokoine	771	Tanzania
27	*L. kirschneri*	Icterohaemorrhagiae	Mwogolo	826	Tanzania
28	*L. kirschneri*	Icterohaemorrhagiae	Mwogolo	845	Tanzania
29	*L. borgpetersenii*	Ballum	Kenya	1605	Tanzania
30	*L. borgpetersenii*	Ballum	Kenya	1610	Tanzania
31	*L. interrogans*	Australis	Lora	1992	Tanzania
32	*L. borgpetersenii*	Ballum	Kenya	2062	Tanzania
33	*L. borgpetersenii*	Ballum	Kenya	2348	Tanzania
34	*L. interrogans*	Australis	Lora	2364	Tanzania
35	*L. borgpetersenii*	Ballum	Kenya	2447	Tanzania
36	*L. kirschneri*	Canicola	Qunjian	2980	Tanzania
37	*L. kirschneri*	Icterohaemorrhagiae	Sokoine	4602	Tanzania
38	*L. borgpetersenii*	Ballum	Kenya	4880	Tanzania
39	*L. borgpetersenii*	Ballum	Kenya	4787	Tanzania
40	*L. borgpetersenii*	Hebdomadis	Kremastos / Hebdomadis	873	Ireland
41	*L. borgpetersenii*	Hebdomadis	Kremastos / Hebdomadis	871	Ireland
42	*L. borgpetersenii*	Sejroe	Saxkoebing	1498	Ireland
43	*L. kirschneri*	Sejroe	Ricardi / Saxkoebing	1499	Ireland
44	*L. kirschneri*	Sejroe	Ricardi / Saxkoebing	1501	Ireland

45	*L. borgpetersenii*	Sejroe	Ricardi / Saxkoebing	1522	Ireland
46	*L. borgpetersenii*	Sejroe	Ricardi / Saxkoebing	1525	Ireland
47	*L. borgpetersenii*	Pomona	Kunming	RIM 139	IRL (Portugal)
48	*L. borgpetersenii*	Pomona	Kunming	RIM 201	IRL (Portugal)
49	*L. borgpetersenii*	Sejroe	Ricardi / Saxkoebing	RIM 156	IRL (Portugal)
50	*L. borgpetersenii*	-	Sokoine	RM1	-
51	*L. kirschneri*	-	Kenya	Njenga	-
52	*L. interrogans*	Grippotyphosa	Ratnapura	GC-1	Andaman
53	*L. interrogans*	Icterohaemorrhagiae	Copenhageni	GC-3	Andaman
54	*L. interrogans*	Grippotyphosa	Ratnapura	TB-6	Andaman
55	*L. interrogans*	Grippotyphosa	Ratnapura	TB-19	Andaman
56	*L. interrogans*	Grippotyphosa	Valbuzii	JAMES	Andaman
57	*L. interrogans*	Icterohaemorrhagiae	Copenhageni	Yasuodamma	Andaman
58	*L. interrogans*	Grippotyphosa	Valbuzii	DS-18	Andaman
59	*L. interrogans*	Grippotyphosa	Valbuzii	DCHCF-30	Andaman
60	*L. interrogans*	Grippotyphosa	Ratnapura	MG-11	Andaman
61	*L. interrogans*	Grippotyphosa	Ratnapura	MG-17	Andaman
62	*L. interrogans*	Grippotyphosa	Ratnapura	MG-23	Andaman
63	*L. interrogans*	Grippotyphosa	-	MG-47	Andaman
64	*L. interrogans*	Sejroe	Saxkoebing	MG-73	Andaman
65	*L. interrogans*	Pomona	-	MG-90	Andaman
66	*L. interrogans*	Grippotyphosa	Ratnapura	MG-100	Andaman
67	*L. interrogans*	Australis	Ramisi	MG-347	Andaman
68	*L. interrogans*	Grippotyphosa	-	MG-79	Andaman
69	*L. interrogans*	Grippotyphosa	Valbuzii	MG-342	Andaman
70	*L. interrogans*	Grippotyphosa	Valbuzii	MG-373	Andaman
71	*L. interrogans*	Australis	Australis	MG-375	Andaman
72	*L. interrogans*	Australis	Australis	MG-392	Andaman
73	*L. interrogans*	Grippotyphosa	Valbuzii	MG-472	Andaman
74	*L. interrogans*	Canicola	Canicola	H-12	South India
75	*L. interrogans*	Autumanalis	-	AUT(N)	South India
76	*L. interrogans*	Canicola	-	PAI	South India
77	*L. interrogans*	Icterohaemorrhagiae	-	Thahkchan	South India
78	*L. interrogans*	Canicola	-	G-1	Central India
79	*L. interrogans*	Canicola	-	G-2	Central India
80	*L. interrogans*	Djasmin	-	G-4	Central India
81	*L. interrogans*	Bataviae	-	G-5	Central India
82	*L. interrogans*	Canicola	-	G-7	Central India
83	*L. interrogans*	Canicola	-	G-8	Central India
84	*L. interrogans*	Canicola	-	G-10	Central India
85	*L. interrogans*	Hebdomadis	-	ALC-1	South India
86	*L. interrogans*	Pomona	-	H-3	South India
87	*L. interrogans*	Pomona	-	H-48	South India
88	*L. interrogans*	Pomona	-	H-61	South India
89	*L. interrogans*	Pomona	-	H-518	South India
90	*L. interrogans*	Pomona	-	H-578	South India
91	*L. interrogans*	Pomona	-	289-M.C.Calicut	South India

2.4. Sources of serum samples

Serum samples from human patients with clinical symptoms of leptospirosis were collected at Nizam Institute of Medical Sciences (NIMS), Hyderabad. Serum samples from normal healthy volunteers were collected at University of Hyderabad, Hyderabad. Bovine serum samples were collected from Ankur, Rajanagaram and Palakonda villages of Mahaboobnagar district of Andhra Pradesh. Anti-LipL32 and anti-LipL41 anitibodies were kindly provided by Dr. David Haake, USA. Anti-HbpA antibodies were raised in rabbit against full-length and 55 kDa recombinant HbpA protein. Anti-sphingomyelinase antibodies (anti-Sph) were raised in rabbit against rSph3 protein that was cloned and expressed using a 638 bp region (nucleotide 511 to 1142) of *sph3* (LA4004).

2.5. Media preparation

EMJH medium preparation was done as per guidelines provided in "International Course on Laboratory Methods for the Diagnosis of Leptospirosis" (KIT, Royal Tropical Institute, The Netherlands).

2.5.1. EMJH - enrichment media

0.23 g of EMJH base was dissolved in 90 mL of pre-autoclaved double distilled water in a 250 mL conical flask and autoclaved at 15 lbs / $inch^2$ pressure for 30 min. Then 10 mL of enrichment was added with the help of a sterile syringe under aseptic conditions and the medium was stirred well. For preparing EMJH semi-solid medium, 0.15 g of Noble agar was added along with EMJH base. Both media were stored at 4ºC for future use.

2.5.2. Iron-free medium

0.23 g of EMJH base was dissolved in 75 mL of pre-autoclaved double distilled water in a 250 mL conical flask and autoclaved at 15 lbs / $inch^2$ pressure for 30 min. In another conical flask, 2 g of BSA was dissolved in 22 mL of double distilled water by gentle stirring on a magnetic stirrer (avoided foaming). Then the following salt solutions, Tween 80 and vitamins (Table 6) were added to the BSA solution and the mix was added

to the EMJH base using 0.2 µm syringe filters under aseptic conditions. The medium was stored at 4ºC for future use.

Table 6. Preparation of albumin fatty acid supplements stock solution.

S. No	Stock solutions (g or mL / 100 mL sterile DDW)	µL / 100 mL of iron free medium
1	$CaCl_2.2H_2O$ + $MgCl_2.6H_2O$ (1.0 g each)	150
2	$ZnSO_4.7H_2O$ (0.4 g)	100
3	$CuSO_4.5H_2O$ (0.3 g)	10
4	Vitamin B12* (0.02 g)	100
5	Glycerol (1 mL)	100
6	Tween 80** (1 mL)	1250
7	Sodium pyruvate (0.11 g)	363
8	Rabbit serum	1000

*All the solutions except vitamin B12 and rabbit serum were autoclaved and stored as aliquots at – 20 ºC.

** Tween 80 was always prepared freshly before use.

2.5.3. Luria - Bertani (LB) media

1 g of tryptone, 0.5 g of yeast extract and 0.5 g of NaCl were dissolved in 100 mL double distilled water and the pH was adjusted to 7.2. The medium was then autoclaved at 15 lbs / $inch^2$ pressure for 15 min and stored at 4ºC for future use. For preparing solid agar, a 1.5 g of bacteriological agar was added to the above ingredients before autoclaving.

2.6. Bacterial growth conditions

Leptospiral serovars were regularly grown in EMJH liquid medium at 30°C. The stock cultures were maintained in EMJH semi-solid medium in screw cap test tubes at 30°C. Growth was monitored regularly under dark field microscope. *Escherichia coli*

strains were grown in LB medium at 37°C added with kanamycin (50 μg / mL) as required by the respective strains.

2.7. Direct demonstration of HbpA expression upon iron-limitation in *Leptospira*.

2.7.1. Establishment of conditions of iron-limitation for the growth of pathogenic *Leptospira* spp.

Growth of *Leptospira* spp. in high- and low-iron conditions was standardized as follows. All glassware was made iron free by soaking with 2% methanolic KOH overnight (O/N) followed by soaking in 8N $HN0_3$ O/N and subsequent washes with double distilled water. Leptospires were initially grown in regular EMJH liquid medium at 30°C until the culture reaches mid log phase. The log phase culture was scaled up gradually over a period of 10 days with the same medium and then divided equally between 2 flasks to represent high-iron and low-iron conditions respectively. Total iron content in low-iron culture was reduced by step wise addition of EDDA. To the low-iron culture, equal volume of iron-free medium and 100 μM EDDA were added and incubated for 24 - 48 h. Then EDDA was increased to a final concentration of 200 μM followed by another 24 - 48 h of incubation. During all the above additions, the cultures were maintained at 30°C. The high-iron culture was grown in regular EMJH liquid medium and additional 4 μg Fe / mL was added in two split doses at corresponding time periods of addition of chelators to low-iron culture. Both cultures were centrifuged at 10,000 rpm for 20 min and low-iron organisms were resuspended in iron-free medium that was pre-incubated overnight with 200 μM EDDA. High-iron organisms were resuspended in EMJH-BSA medium with 10 μg iron / mL and were incubated for 5½ h at 37°C before harvesting.

2.7.2. Preparation of whole cell sonicate

The cultures were harvested at 10,000 rpm for 20 min, washed thrice and were re-suspended in 200 μL of 10 mM Tris-HCl (pH 8.0) and sonicated for about 5 min (20 sec pulse at 20 Hz in Vibra cell sonicator, USA). 1% SDS was added to the sonicated

samples and incubated at 37°C O/N. Samples were centrifuged at 10,000 rpm for 10 min to remove the cell debris.

2.7.3. Preparation of outer membrane proteins (OMPs) (Haake *et al*., 1998)

Preparation of solutions

1) Wash buffer**:** 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$.

2) Lysis buffer: 10 mM Tris-HCl buffer (pH 8.0) with 150 mM NaCl and 1 mM EDTA.

3) Solubilisation buffer: 10 mM Tris-HCl buffer (pH 8.0) containing 0.6 % SDS.

4) Pre-condensation of Triton X-114 (Bordier, 1981): 1 mL of Triton X-114 was mixed with 500 mL of 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl. After complete dissolution at 4ºC, the clear solution was incubated at 37ºC O/N. Condensation of detergent occurred and mixture separated into aqueous and detergent phases. The aqueous phase was discarded and replaced by same volume of above buffer. Condensation was repeated thrice and stored at 4ºC.

The OMPs of *Leptospira* serovars grown under high- and low-iron conditions were prepared by Triton X-114 detergent extraction method. The cultures were harvested, washed thrice and incubated with O/N shaking at 4°C in 2% Triton X-114 in 1 mL of lysis buffer. The insoluble material (cell pellet / cytoplasmic cylinder) was removed by centrifugation at 17,000 x g for 10 min and 20 mM $CaCl_2$ was added to the supernatant. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 15 min at 2000 x g. The bottom detergent phase (OMP fraction) was washed thrice with lysis buffer and the top aqueous phase (periplasmic protein fraction) was washed thrice with 2% Triton X-114. Both the fractions were subjected to acetone precipitation at - 20ºC. The OMP fraction was solubilised in solubilisation buffer and analyzed by 5 – 20% gradient SDS-PAGE.

2.7.4. Precipitation of proteins by acetone

Five volumes of ice-cold acetone was added to the protein sample and incubated O/N at - 20°C. It was centrifuged at 12,000 rpm for 20 min at 4°C and washed once with acetone. The protein pellet was dissolved in appropriate buffer, stored at - 20°C for further use.

2.7.5. Protein estimation

Total protein concentration in the sample was estimated by BCA protein assay reagent kit as per manufacturer's instructions.

2.7.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

Stock solutions

1) Acrylamide and N, N'-bisacrylamide mix (30:0.8): 30 g of acrylamide and 0.8 g of bisacrylamide were dissolved in 60 mL of water and made up to 100 mL.

2) Resolving gel buffer: Tris-HCl (1.5 M, pH 8.8) with 0.4% SDS.

3) Stacking gel buffer: Tris-HCl (0.5 M, pH 6.8) with 0.4% SDS.

4) Ammonium per sulfate (APS): 10% APS solution was prepared freshly in double distilled water.

5) Sample buffer (2X): 0.125 M Tris-HCl (pH 6.8) containing 4% SDS, 20% glycerol and 0.002% bromophenol blue.

6) Running buffer: Tris-glycine buffer (25 mM Tris, 250 mM glycine and 0.1% SDS), pH 8.8.

7) Staining solution: 0.25% Coomassie Brilliant Blue R-250 in 60% methanol and 10% glacial acetic acid.

8) Destaining solution: 10% Methanol and 10% glacial acetic acid.

Table 7. Preparation of resolving gel mix

Ingredients	Volume of the ingredients (mL)		
	Gradient gel		10%
	5%	20%	
Acrylamide: Bisacrylamide	2.75	10.60	10.60
Resolving gel buffer	4.00	4.00	8.00
Double distilled water	9.30	1.40	13.40
Ammonium per sulfate	0.08	0.08	0.16
TEMED	0.008	0.008	0.008
Total volume	**16.138**	**16.088**	**32.168**

The resolving gel was prepared as 5 - 20% gradient gel or 10% gel using the recipe as given above. The gradient was prepared using a gradient maker. The resolving gel was allowed to polymerize and then the stacking gel was poured over it after appropriately positioning the comb.

Table 8. Preparation of 5% stacking gel mix

Components	Volume (mL)
Acrylamide: Bisacrylamide	1.5
Stacking gel buffer	2.5
Double distilled water	6.0
10% Ammonium per sulfate	0.03
TEMED	0.01
Total	**10.04**

About 30 µg of protein was loaded on to minigels and 75 µg of protein was used for gradient standard gels. Equal volumes of the protein samples and 2X sample buffer were mixed, boiled for 10 min and centrifuged for 10 min at 10,000 rpm to remove any insoluble material. The clear supernatant was loaded onto gel and electrophoresis was carried out at 25 mA constant current using standard Hoefer electrophoresis unit (SE600 series). The electrophoresis was allowed to run until the tracking dye was run out of the gel, followed by electrophoresis for an additional 15 min. The gel was stained with Coomassie Blue for 2 - 4 h and then destained O/N.

2.7.7. Western blotting analysis (Towbin *et al.*, 1979)

Preparation of stock solutions

1) Transblot buffer (10X): Stock solution was prepared by dissolving 250 mM Tris and 1.3 M glycine in 400 mL of double distilled water. Working solution was prepared by mixing 200 mL of 10X stock solution and 400 mL of methanol in 1.4 L of double distilled water.

2) Tris buffer saline (TBS): 50 mM Tris HCl (pH 8.0) containing 150 mM NaCl in 1.0 L of double distilled water.

3) Tris buffer saline – Tween solution (TBS-T): TBS containing 0.05% Tween – 20.

4) Ponceau S stain: Ponceau S, trichloroacetic acid and sulfosalicylic acid were mixed in 2:30:30 (w/v) ratio and the final volume was made up to 100 mL with double distilled water. One part of stock solution was diluted with 9 parts of deionized water to make a working solution.

The proteins resolved on polyacrylamide gel were electrophoretically transferred on to nitrocellulose membrane at 30 V constant voltage O/N or 60 V for 2½ h, employing transblot buffer using Broviga transfer apparatus. After the transfer, proteins were visualized by Ponceau S stain to check the protein transfer efficiency and also for marking the protein molecular weight marker. The membrane was then blocked for 2 h using 5% non fat milk solution (NFM) dissolved in TBS-T. The membrane was then washed thrice with TBS and incubated O/N with appropriate antiserum (1:600 for anti-HbpA / anti-Sph / anti-human transferrin / clinical serum samples; 1: 10000 for anti-LipL32 and 1: 8000 for anti-LipL41 antibodies) diluted in TBS-T containing 1% NFM at 4°C. Later, the membrane was washed 4 times with TBS and incubated in 1:500 dilution of anti-IgG ALP conjugate in TBS-T containing 1% NFM at room temperature for 1½ h. Then, the blot was washed thoroughly with TBS and developed using ready to use BCIP – NBT.

For identification of transferrin-binding proteins, nitrocellulose membrane was incubated with TBS containing human transferrin (0.1 mg / mL) and subsequently detected its binding with goat anti-human transferrin antibodies.

2.7.8. Hemin-agarose affinity chromatography (Lee, 1992)

Preparation of solutions

1. Wash buffer**:** 25 mM Tris-HCl (pH 7.4) containing 100 mM NaCl.
2. TN buffer: 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl.
3. TNE buffer: TN buffer containing 10 mM EDTA.

Hemin-agarose batch affinity chromatography was performed as described below. 200 µL of hemin-agarose was washed thrice with 1 mL of wash buffer, with

centrifugation done at 750 x *g* for 5 min. The hemin-agarose beads were incubated with OMP extract for 60 min at 37ºC with gentle mixing. Agarose beads were then separated by centrifugation at 2,000 rpm for 10 min, and un-bound proteins were removed by extensive washing with the following buffers; thrice with TNE buffer and twice with TN buffer. The beads were then suspended in 40 mL of sample buffer (2X) containing β-mercaptoethanol (1% v/v) and heated at 100ºC for 5 min and centrifuged for about 10 min. The supernatant was analyzed by 10% SDS-PAGE.

2.7.9. Confocal microscopy and immunofluorescence studies (Cullen *et al*., 2005)

Preparation of reagents

1) Anti-HbpA immunoglobulins: Anti-HbpA immunoglobulins were prepared by 33% ammonium sulfate precipitation of polyclonal rabbit antiserum raised against rHbpA followed by dialysis to remove the ammonium sulfate.

2) Propidium iodide solution: 50 µg of propidium iodide was dissolved in 5 mL of double distilled water and stored in aliquots at 4ºC.

3) PBS-T: PBS containing 0.05% Tween-20.

Confocal microscopy was performed using a Leica TCS SP2 AOBS microscope. *Leptospira interrogans* serovar Lai cells grown under high- and low-iron conditions were harvested at 10,000 rpm for 20 min, washed thrice with PBS (pH 7.2), and re-suspended in PBS with 2.5% BSA to a cell density equivalent to McFarland II. Then a thin smear was prepared on a slide, allowed to air dry, and then heat fixed by quickly passing the slide through the flame of a Bunsen burner twice. The smear was then treated with ice-cold methanol at - 20°C for 40 min and then blocked with 5% BSA for 1½ h. After four washes with PBS, anti-HbpA immunoglobulins were added to the smear at a dilution of 1:100 to the fixed leptospires and incubated O/N at 4°C. After four washes with PBS-T, the slide was incubated with a 1:500 dilution of goat anti-rabbit FITC conjugate for 1½ h at room temperature and then subjected to four washes with PBS to remove the unbound conjugate. Propidium iodide was applied as a counter stain. A drop of 90% glycerol was added to the slide to keep it moist, overlaid with a cover slip, and sealed. Then the fluorescence was visualized in the confocal microscope. Identical smear preparations of high- and low-iron organisms from the same batch of cells described above were

incubated with anti-LipL41 antibodies and used as a control. The excitation wavelength for FITC is 500 nm, with emission at 535 nm, while the excitation and emission wavelengths for propidium iodide are 600 and 732 nm, respectively.

2.8. Molecular biology studies for cloning and expression of rHbpA$_{55}$ (Sambrook *et al*., 1989)

Cloning and expression studies were done following standard molecular biology protocols. pET-28a(+) vector system (Fig. 9) was used for cloning and *E. coli* BL21 (DE3) was used as host for expression studies.

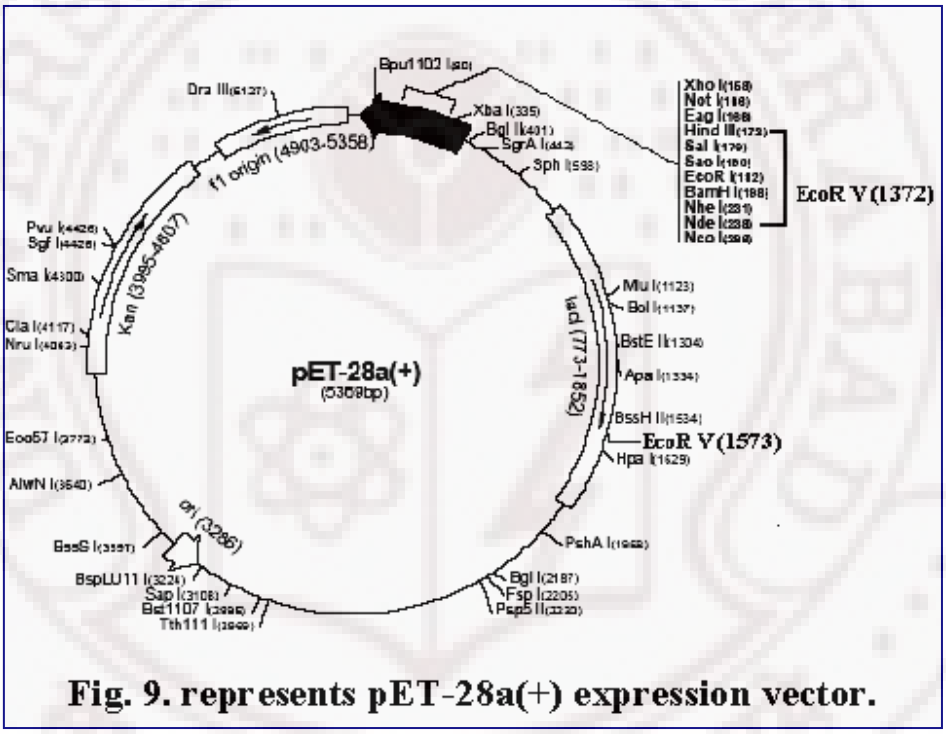


Fig. 9. represents pET-28a(+) expression vector.

2.8.1. Isolation of leptospiral genomic DNA (Marmur, 1961)

Preparation of solutions

1) TE buffer: 10 mM Tris-HCl; pH 8.0 containing 1 mM EDTA

2) Lysozyme (10 mg / mL stock): Dissolved 50 mg of lysozyme in 5 mL double distilled water, aliquoted and stored at - 20°C.

3) Proteinase K (10 mg / mL stock): Dissolved 50 mg of proteinase K in 5 mL double distilled water, aliquoted and stored at - 20°C.

Leptospiral genomic DNA was isolated according to standard detergent-proteinase K lysis method. 10 mL culture was harvested at 6,000 rpm for 15 min and washed thrice with TE buffer. The cell pellet was re-suspended in 400 µL of TE buffer and then treated with 50 µL of lysozyme for 1 h at 37°C. The clear solution was then treated with 6 µL of proteinase K, 70 µL of 10% SDS and incubated in a water bath at 65°C for 10 min. The sample was then equilibrated to room temperature and added 100 µL of 5 M NaCl. To this, an equal volume of phenol:chloroform (1:1) was added, mixed well by gentle inversion of microfuge tube and centrifuged at 12,000 rpm for 15 min at 4°C. The top aquoeous layer was separated and phenol:chloroform extraction was repeated twice, followed by the addition of an equal volume of isopropanol and incubated the mixture at room temperature for 30 min to precipitate the DNA. The DNA was pelleted by centrifugation at 12,000 x g for 15 min at 4°C, washed in 1 mL of cold 70% ethanol. DNA pellet was air dried at room temperature and dissolved in TE buffer. DNA concentration was estimated spectrophotometrically by measuring the $OD_{280\ nm}$.

2.8.2. Agarose gel electrophoresis

Preparation of reagents

1) TAE buffer: Dissolved 4.84 g Tris, 1.14 mL glacial acetic acid and 20 mL 0.5 M EDTA in 1 L of double distilled water and pH was adjusted to 8.0.

2) Ethidium bromide: 1 mg / mL stock solution was prepared and added to the gels to give a final concentration of 0.1 µg / mL.

3) Gel loading buffer: 0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 40 % (w/v) sucrose in water.

Genomic DNA and PCR amplified products were subjected to electrophoretic separation using 0.8% and 1.2% agarose gels, respectively. The gels were prepared by adding appropriate amounts of agarose into TAE buffer and subsequent boiling in a microwave for complete dissolution. The DNA samples were prepared by adding 6X gel loading buffer (6:1 - DNA:loading buffer) and loaded onto the gel. The DNA size standards loaded onto gel included 8 µL of 1 kb ladder or λ DNA, *EcoRI-HindIII* double digest (0.5 µg / µL stock). Samples were subjected to electrophoresis at 100 V and the DNA was visualised in a UV transilluminator.

2.8.3. PCR amplification of *hbpA*

PCR amplification of 1449 bp fragment of *hbpA* gene was done using genomic DNA of *L. interrogans* serovar Lai strain Lai as template. PCR was performed in a final volume of 20 µL, using 50 – 100 ng of template DNA. The reaction mixture contained 2 µL of 10X Taq buffer with KCl, 200 µM of each dNTP, 0.5 U Taq DNA polymerase, 0.15 mM $MgCl_2$, and 100 ρmoles of each oligonucleotide primer (Table 9). A negative control without template DNA was included.

Table 9. Primers used in this study

Gene	Primer sequence (5'→ 3')
hbpA (full-length)	**H 1**: GGGAATTC**CAT^ATG**TCATCCAACCATTCGATG – *NdeI* **H 2**: CCC**A^AGCTT**TTAAAACTGGGCCGAGAATC – *HindIII*
hbpA (1449 bp)	**H3:** GGGAATTC**CAT^ATG**GAATTCAATACCACAGCCAACATGGG - *NdeI* **H4:** CCC**A^AGCTT**TTAAAACTGGGCCGAGAATC - *HindIII*

PCR was done with an initial denaturation step (5 min, 95°C) followed by 30 cycles of amplification (1 min at 95°C, 1 min at 50°C and 2 min at 72°C) and a final extension for 15 min at 72°C done in a PTC-200 thermal cycler (MJ research, USA). The PCR products were subjected to electrophoresis in 1% agarose gel and visualized under UV light using ethidium bromide staining. The PCR amplified *hbpA* (1449 bp) product was gel purified using Biogene DNA gel extraction kit according to manufacturer's recommendations and kept at - 20°C.

2.8.4. Cloning experiments

a) Restriction digestion of vector and insert

1 µg of pET-28a(+) plasmid DNA and 1449 bp PCR product were individually subjected to double digestion. 2 units of *NdeI* and *HindIII* restriction enzymes were added in a final volume of 50 µL containing 5 µL of 10 X buffer R with BSA, and incubated at 37°C for 16 h. Both reactions were terminated by heat inactivation at 65°C for 10 min. 2 µL sample was analyzed on 1% agarose gel. Digested plasmid and insert DNA were purified using mini-elute reaction clean up kit (Qiagen, Germany).

b) Ligation of vector and insert DNA

Digested vector and insert DNA were quantified spectrophotometrically by measuring $OD_{280\ nm}$. Ligation was done using vector and insert DNA in molar ratio of 3:1 using the following formula.

$$\text{Concentration of the insert (ng)} = \frac{\text{ng of the insert x Size of the insert (kb)}}{\text{Size of the vector (kb)}} \text{ x Molar ratio}$$

The ligation reaction was carried out in a total reaction volume of 10 µL containing 100 ng of vector DNA, 35 ng of insert DNA, 1.5 µL of 10X ligase buffer, 1 µL of T4 DNA ligase (5 U / µL) and sterile double distilled water. The reaction mixture was incubated at 16°C O/N. After completion of the reaction, the sample was subjected to 65°C for 10 min and then stored at - 20ºC or used for transformation immediately.

2.8.5. Bacterial transformation experiments

The recombinant plasmid harboring 1449 bp *hbpA* gene was transformed into *E. coli* DH5α by $CaCl_2$ method.

a) Preparation of *Escherichia coli* competent cells

A single colony of *E. coli* DH5α was picked with a sterile inoculation loop and inoculated into 5 mL of LB broth under aseptic conditions. The culture was incubated O/N at 37ºC in an orbital shaker.

100 mL of LB medium without antibiotics was inoculated with 5 mL of O/N grown culture. When the cell density was (OD_{600nm}) 0.6, it was harvested by centrifugation at 3,000 rpm for 10 min at 4°C. The cell pellet was re-suspended in 15 mL of 0.1 M $CaCl_2$ and incubated in ice for 30 min. The cell suspension was centrifuged at 3,000 rpm for 10 min; the cell pellet re-suspended in 1 mL of 0.1 M $CaCl_2$ and then dispensed as 200 µL aliquots in 1.5 mL eppendorf tubes. All the operations were performed under sterile conditions at 4°C.

b) Transformation by $CaCl_2$ method

The recombinant plasmid was used for transformation into *E. coli* DH5α. 10 µL of recombinant plasmid was added to 200 µL of competent cells, mixed gently and

incubated on ice for about 30 min. The cells were then subjected to heat shock at 42°C in water bath for 90 sec and transferred to ice for 2 min. 800 µL of LB broth was added to the mixture and cells were incubated at 37°C for 1 h in a shaking incubator at 225 rpm (Sambrook *et al.,* 1989). The cells were plated out on LB agar plates supplemented with kanamycin (50 µg / mL). The plates were incubated at 37°C O/N. Isolated colonies were picked and tested for the presence of insert by plasmid digestion and colony PCR.

2.8.6. Isolation of plasmid DNA: Miniprep method

Preparation of solutions

1) Solution I: 50 mM glucose was dissolved in 50 mL of 25 mM Tris-HCl (pH 8.0) containing 10 mM EDTA. The pH was adjusted to 8.0 and stored at 4°C.

2) Solution II: 0.2 N NaOH solution containing 1.5% SDS was prepared fresh.

3) Solution III: Prepared by mixing 5M potassium acetate (pH 5.2), glacial acetic acid and double distilled water in ratio of 60.0:11.5:28.5 (v/v).

A single colony of *E. coli* strain DH5α, obtained as described above, was inoculated into 5 mL of LB medium containing kanamycin and incubated overnight with shaking at 37°C. An aliquot of 1.5 mL of the culture was transferred to an eppendorf tube and centrifuged for 5 min at 6,000 rpm at 4°C. The supernatant was removed by aspiration. The pellet was re-suspended in 100 µL of Solution I by vortexing and incubated on ice for 5 min. Then 200 µL of freshly prepared Solution II was added, the contents were mixed well, and then incubated on ice for 5 min. The solution was then neutralized by adding 150 µL of ice-cold Solution III, mixed by inversion and stored on ice for 5 min. Then the cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and DNase free RNase was added at a final concentration of 10 µg / mL and incubated at 37°C for 30 min. After the RNase treatment, the suspension was extracted twice with addition of equal volumes of phenol:chloroform (1:1) mix, mixed by complete inverting of tube and subsequent centrifugation at 12,000 rpm for 15 min. The plasmid DNA in the aqueous phase was precipitated with 2 volume of isopropanol at room temperature for 10 min and centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed with 70% ethanol, air-dried, and dissolved in 20 µL of TE buffer and stored at –20°C.

2.8.7. Analysis of recombinants by colony PCR

About 8 transformed colonies were picked, streaked on LB agar plate having kanamycin and allowed to grow for exactly 12 h. The colonies grown were picked serially, numbered and used directly for PCR analysis using gene specific primers (Table 9). Recombinant plasmid from a positive clone was isolated and again transformed into *E. coli* BL21 (DE3) cells as described above for expression studies.

2.8.8. Expression of recombinant $HbpA_{55}$ protein in *E. coli*

Escherichia coli strain BL21 (DE3) containing the expression vector harboring the recombinant *hbpA* gene was grown in LB medium supplemented with kanamycin at 37°C in an orbital shaker until the OD_{600nm} of the culture reached 0.6. Then an aliquot of culture was removed, which represented un-induced cells and the remaining culture was induced with 1 mM IPTG and allowed to grow for an additional two hours. Both un-induced and induced cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C, washed thrice and subjected to sonication for 2 min (20 sec pulse in Vibra Cell sonicator). The pellet and supernatant separated by centrifugation at 10,000 rpm for 20 min were analyzed by SDS-PAGE using a 10% gel.

2.8.9. Purification of $rHbpA_{55}$ protein

The expressed $rHbpA_{55}$ was located in the insoluble inclusion bodies fraction as deduced by 10% SDS-PAGE. Two methods were adopted for purification of $rHbpA_{55}$.

a) Purification of $rHbpA_{55}$ by gel elution (Yeruva *et al.*, 2006)

Preparation of solutions

1) Elution buffer: 25 mM Tris-HCl (pH 8.8) buffer containing 5% glycerol, 1% SDS and 0.24 mM glycine.

The insoluble inclusion bodies containing the expressed $rHbpA_{55}$ protein was separated on 10% preparative SDS-PAGE and $rHbpA_{55}$ gel slice was excised, crushed with the help of a gel crusher and eluted using elution buffer and subsequent incubations at 37°C. The eluted protein was evaluated again by SDS-PAGE to confirm the purity of the band.

b) Affinity chromatography using Ni-NTA His bind column: purification under denaturing conditions

Preparation of solutions

1) Buffer A: 6 M Guanidium HCl in 20 mM Tris-HCl (pH 7.9) containing 0.5 M NaCl and 10% Glycerol.

2) Buffer B: 8 M urea in 100 mM Tris-HCl containing 100 mM NaH_2PO_4 (pH 6.3)

3) Buffer C: 8 M urea in 100 mM Tris-HCl containing 100 mM NaH_2PO_4 (pH 5.9)

4) Buffer D: 8 M urea in 100 mM Tris-HCl containing 100 mM NaH_2PO_4 (pH 4.5)

Purification of $rHbpA_{55}$ using Bug Buster Ni-NTA His bind resin under denaturing conditions was done as per manufacturer's instructions with few modifications. A column with 2.0 mL bed volume of Ni-NTA His bind resin was packed and charged by passing thrice with 2 bed volumes of 1 x charge buffer without denaturant. The resin was then equilibrated with 5 bed volumes of buffer B before loading the sample. Then the insoluble inclusion bodies were dissolved in buffer A for 5 – 10 min, incubated on ice for another 30 min and centrifuged at 20,000 x g for 20 min. The soluble supernatant was loaded on to the pre-charged column and incubated for 1 h at room temperature. Then the resin was washed with 5 bed volumes of buffer B. The bound His-$HbpA_{55}$ fusion protein was eluted using 4 bed volumes of buffer C and D.

All the fractions were concentrated using Amicon ultrafiltration tubes. After protein estimation, they were analyzed by SDS-PAGE using a 10% gel.

2.8.10. Antisera against $rHbpA_{55}$

Antiserum against $rHbpA_{55}$ was prepared by immunizing rabbits subcutaneously and intramuscularly with 100 µg of purified $rHbpA_{55}$ mixed with Freund's complete adjuvant. The secondary immunization was done using 50 µg of purified $rHbpA_{55}$ mixed with Freund's incomplete adjuvant. The rabbit was bled 2 weeks after secondary immunization and serum was separated and stored in - 80ºC in separate aliquots for further use.

2.8.11. Purification of anti - HbpA antibodies

Preparation of solutions

1) Tris buffered saline (TBS): 25 mM Tris containing 0.15 M NaCl (pH 7.2).
2) Regeneration buffer: 0.1 M Glycine; pH 2.8

Selective removal of anti-*E. coli* immunoglobulins from polyclonal anti-HbpA antisera was done using the commercial Immobilized *E. coli* lysate kit as per manufacturer's instructions. The immobilized *E. coli* lysate column and buffers were equilibrated to room temperature. The column was equilibrated with 4 mL of TBS. 2 mL of serum sample was loaded onto the column, 20 mL TBS was added and fractions were collected. $OD_{280\ nm}$ of 1 mL fractions was collected and fractions were mixed for further use. The column was regenerated by washing with at least 10 mL of regeneration buffer. After regeneration, the column was washed immediately with 10 mL of TBS containing 0.02% sodium azide. The column was stored upright at 4°C by placing the cap with > 1 mL of storage solution remaining above the resin bed.

2.9. Evaluation of diagnostic potential of HbpA

2.9.1. Screening of serum samples from patients with leptospirosis

2.9.1.1. LeptoTek Dri-Dot test (Biomerieux, The Netherlands)

The test was performed as per the manufacturer's instructions employing 10 µL of human clinical serum sample. Aggregation of latex particles of the test dot reveals agglutination by *Leptospira*-specific antibodies present in serum sample. The results were interpreted as positive, if fine granular agglutination settled at the edges of the blue droplet in about 30 seconds and as negative, if the blue suspension remained homogeneous. Care was taken to record all the results within 30 sec.

2.9.1.2. IgM ELISA test (Pan-Bio, Australia)

IgM ELISA using *Leptospira biflexa* serovar Patoc antigen attached to polystyrene surface of micro well test strips was performed according to manufacturer's instructions employing 10 µL of human clinical serum sample. After addition of serum, plates were incubated for 30 min at 37°C. HRP conjugated anti-human IgM and TMB were provided by the manufacturer and they were used as per instructions. Then the plate

was read at $OD_{450\ nm}$ and the readings were interpreted in terms of Pan-Bio units calculated as per manufacturer's instructions. Positive control serum, negative control serum and cut-off calibrator, provided by the manufacturer were used for calculation of Pan-Bio units from the observed absorbance. Samples were recorded as positive if the number of Pan-Bio units were more than 11.

2.9.1.3. Microscopic agglutination test (MAT) (Cole *et al*., 1973)

MAT was performed employing the following representative leptospiral cultures: a) *L. interrogans* – serovar Pomona strain Pomona, serovar Lai strain Lai, serovar Australis strain Ballico, serovar Rachmati strain Rachmat, serovar Hebdomadis strain Hebdomadis, serovar Hardjo strain Hardjoprajitno, b) *L. kirschneri* serovar Grippotyphosa strain Moskva V, c) *L. borgpetersenii* – serovar Tarassovi strain Perepelitsin, serovar Ballum strain MUS127, serovar Javanica strain Poi, d) *L. meyeri* serovar Ranarum strain ICF and e) *L. biflexa* serovar Patoc strain Patoc – I. All the cultures were grown in EMJH liquid medium and diluted to a density equivalent to McFarland I for use in MAT. Care was taken to ensure that there was no auto agglutination or clumping in the culture.

In a microtitre plate, 50 µL of serum at dilutions ranging from 1:50 to 1:3200 in 0.1 M phosphate buffered saline (pH 7.2) was added. Then 50 µL of specific serovar was added to all the serum dilutions. The antigen control included 50 µL of antigen without addition of antibody. The plates were mixed thoroughly on a micro shaker and incubated for 4 h at 37ºC. For observation, one drop of mixture from each well was transferred with a micropipette to a microscopic slide and examined for agglutination under dark field microscope.

The end point titre was the highest dilution of serum in which 50% of leptospiral cells were agglutinated or 50% reduction in total number of leptospiral cells was observed as compared to the control. Serum samples showing end point titres ≥1:100 were considered as positive for leptospirosis (Plank & Dean, 2000).

2.9.1.4. Dot blot analysis

Purified rHbpA$_{55}$ was used as the antigen in dot blot analysis. 5 µg of rHbpA$_{55}$ was spotted on nitrocellulose membrane and after blocking the membrane with 5% non-fat milk powder in TBS-T, human serum (1:600 dilution) was added and incubated overnight. Then anti-human IgG ALP- conjugate at 1:500 dilution was added, followed by development of the blot with the ready-to-use BCIP - NBT substrate. The blots were then scanned and analyzed using Image J, NIH software. The densitometric scan data of the dot blots are presented as mean of triplicate values. The data obtained were analyzed using the Mann - Whitney two - tailed test and ANOVA. $P < 0.05$ was considered as statistically significant.

2.9.2. Enzyme-linked immunosorbent assay (ELISA) for the screening of anti-HbpA antibodies in serum samples from patients with leptospirosis

2.9.2.1. Standardization of sandwich ELISA

The working concentrations of antigen and antibodies of anti-HbpA antibodies were determined by checkerboard titration using ELISA (Senthilkumar *et al*., 2007) prior to perform sandwich ELISA.

a) Determination of concentration of antigen and antibody by direct ELISA

Preparation of solutions

1) 0.1 M Na_2CO_3 – $NaHCO_3$ buffer: Dissolved 0.293 g of sodium bicarbonate and 0.159 g of sodium carbonate in 100 mL of double distilled water and adjusted the pH to 9.2. Then the solution was autoclaved and stored at 4°C.

2) 10 x PBS: Dissolved 80 g of NaCl, 2 g of KH_2PO_4, 29 g of Na_2HPO_4 and 2 g of KCl in 1 L of double distilled water and autoclaved. It was diluted 10 times to get 1 x PBS.

3) PBS-T: 1 x PBS containing 0.05% Tween 20.

4) Blocking solution: 1 x PBS containing 5% BSA.

The working concentrations of anti-HbpA antibodies and rHbpA$_{55}$ antigens were determined by checkerboard titration. A 96 - well flat - bottom microtiter plate was coated with different concentrations of rHbpA$_{55}$ antigen such as 0.1, 0.5, 1.0, 1.5 and 2.0 µg of in 100 µL of carbonate buffer and incubated at 37°C for 3 h, followed by overnight incubation at 4°C in a humid box chamber. Between all subsequent incubation steps, the

plate was washed three to four times with PBS-T and all dilutions were made in PBS. The unbound sites of wells were blocked by incubation with 300 µL of blocking solution at 37°C for 2 h. The plate was then washed, and incubated with 100 µL of different dilutions of anti-HbpA antibodies (1:50, 1:100, 1:200, 1:400 and 1:800) for 2 h at 37°C. After washing the unbound antibody, 100 µL of goat anti-rabbit / anti-human ALP secondary antibody (1:3000) was added and incubated for 1½ h at 37°C. Color development was performed by the addition of 100 µL of freshly prepared para-Nitro Phenyl Phosphate (pNPP) substrate solution (ALP substrate kit; Bio-Rad, USA), incubated at room temperature for 15 min and the absorbance at $OD_{405\ nm}$ was read with an ELISA reader.

b) Determination of cross-reactivity of anti-HbpA antibodies

A 96 - well flat - bottom microtiter plate was coated with either 5 µg of antigens from commonly infected human pathogenic bacteria such as *Escherichia coli* strain ATCC25922, *Proteus vulgaris, Staphylococcus aureus* strain ATCC29213, *Pseudomonas aeruginosa strain* ATCC27853 and *Klebsiella pneumoniae* or 1 µg of pure $rHbpA_{55}$ antigen in 100 µL of carbonate buffer and incubated at 37°C for 3 h, followed by overnight incubation at 4°C in a humid box chamber. Between all subsequent incubation steps, the plate was washed three to four times with PBS-T and all dilutions were made in PBS. The unbound sites of wells were blocked by incubation with 300 µL of blocking solution at 37°C for 2 h. The plate was then washed, and incubated with 100 µL of diluted anti-HbpA antibodies (1:100) for 2 h at 37°C. After washing the unbound antibody, 100 µL of goat anti-rabbit ALP secondary antibody (1:3000) was added and incubated for 1½ h at 37°C. Color development was performed by the addition of 100 µL of freshly prepared para-Nitro Phenyl Phosphate (pNPP) substrate solution (ALP substrate kit; Bio-Rad, USA), incubated at room temperature for 15 min and the absorbance at $OD_{405\ nm}$ was read with an ELISA reader.

2.9.2.2. Sandwich ELISA (He *et al*., 2007)

A 96 - well flat - bottom microtiter plate was coated with 1:50 dilutions of anti-HbpA in 100 µL of carbonate buffer and incubated at 37°C for 3 h, followed by overnight incubation at 4°C in a humid box chamber. Between all subsequent incubation steps, the

plate was washed three to four times with PBS-T and all dilutions were made in 1X PBS containing 1% BSA. The unoccupied sites of wells were blocked by incubation with 300 µL of blocking solution at 37°C for 2 h. The plate was then washed, and incubated with 1 µg of purified $rHbpA_{55}$ in 100 µL of PBS at 37°C for 2 h. After the plates were rinsed, 100 µL of appropriately diluted human serum or bovine serum (1:400) was added and the mixture was incubated for 2 h at 37°C. The plate was then washed and further incubated with either 100 µL of diluted ALP-conjugated rabbit anti-human (1:3000) or 1:500 dilution of anti-bovine ALP secondary antibody for 1½ h at 37°C. Color development was performed by the addition of 100 µL of freshly prepared para-Nitro Phenyl Phosphate (pNPP) substrate solution (ALP substrate kit; Bio-Rad, USA), incubated at room temperature for 15 min and the absorbance at $OD_{405\ nm}$ was read with an ELISA reader.

2.9.2.3. Statistical analysis

The relative sensitivity, specificity and accuracy of the test were determined using MAT as the gold standard (Senthilkumar *et al*., 2007) described below:

$$\text{Sensitivity} = a/(a+c) \times 100,$$

where 'a' is the number of serum samples positive by the ELISA and MAT, 'c' the number of serum samples positive by MAT but negative by ELISA.

$$\text{Specificity} = d/(b+d) \times 100$$

where'd' is the number of serum samples negative by ELISA and MAT, 'b' the number of serum samples negative by MAT but positive by ELISA.

$$\text{Accuracy} = a+d/(a+b+c+d) \times 100$$

An intuitive method for calculating predictive values for positive and negative test results was done as below (Jacobson, 1998):

Positive predictive value = $a/(a+b) \times 100$

Negative predictive value = $d/(c+d) \times 100$

2.10. Iron levels and expression of the sphingomyelinase(s) in *Leptospira interrogans* serovar Lai

2.10.1. *In silico* analysis of the leptospiral hemolysins

Nucleotide and amino acid sequences were analyzed using standard SWISS-PROT bioinformatics and proteomics tools. BLASTN and BLASTX were used to search the genome of *Leptospira interrogans* serovar Lai. CLUSTALW was used for multiple sequence alignment. Sequence analysis and construction of phylogenetic tree for proteins was done using CLUSTALX and MEGA 3.1 programs. Sequence alignment was done using GENEDOC software.

2.10.2. Detection of sphingomyelinase(s) in outer membrane vesicles (OMVs)

2.10.2.1. Isolation of OMVs (Balsalobre *et al.*, 2006)

OMVs were isolated from spent growth medium of *L. interrogans* serovar Lai cultures grown under high- and low-iron conditions. The spent growth medium was obtained by removal of organisms from the culture by centrifugation at 10,000 rpm for 20 min at 4°C. Sequentially, the spent growth medium was filtered through a 0.2 µm-pore-size syringe filter. Vesicles were collected by subjecting to ultracentrifugation at 1,45,000 x *g* for 3 h at 4°C in Beckman L8-80M Ultracentrifuge. The supernatant was carefully discarded and the viscous pellet containing the OMVs was washed, re-suspended in Tris-HCl buffer (20 mM, pH 8.0) and stored at - 20°C till use.

2.10.2.2. Transmission Electron Microscopy (TEM) of OMVs (Horstman & Kuehn, 2000)

TEM was performed at Acharya N.G. Ranga Agricultural University, Hyderabad, using standard protocol. A drop of OMVs suspension prepared from low-iron culture of serovar Lai was placed over the carbon coated grid and allowed to stand for 5 min. Then the grid was washed with double distilled water and stained with 1% uranyl acetate. The negatively stained OMVs were visualized using Hitachi (Model H-7500) transmission electron microscope.

2.10.2.3. SDS-PAGE and western blotting analysis of OMVs

OMVs of *L. interrogans* serovar Lai grown under high- and low-iron conditions were separated by 5 – 20% gradient SDS-PAGE, electrophoretically transferred on to nitrocellulose membrane and probed with anti-Sph / anti-LipL32 / anti-HbpA antibodies. For detailed procedure, refer methodologies 2.7.6 and 2.7.7.

2.10.2.4. Assay of hemolytic activity of OMVs (Oscarsson *et al*., 1999)

Quantitative hemolytic assay was performed with OMVs of *L. interrogans* serovar Lai as per published protocols. Aliquots of OMVs equivalent to a total protein concentration of 100 µg were taken for each of the following experiments. To 50 µl of 20% sheep RBC suspension (prepared in 0.9% NaCl) added an equal volume of (a) 0.9% NaCl (negative control with zero lysis), (b) double distilled water (positive control showing 100% lysis), (c) & (d) suspension of OMVs from high- & low-iron cultures, (e) & (f) suspension of OMVs from high- & low-iron cultures plus $CaCl_2$ (added to a final concentration of 10 mM) and (g) & (h) suspension of OMVs from high- & low-iron cultures, pre-incubated for 1h with anti-Sph antibodies (at 1:200 dilution). The samples in microfuge tubes were incubated for 60 min at 37°C, followed by the addition of 100 µl of ice-cold 0.9% NaCl. The microfuge tubes were centrifuged at 400 x *g* for 15 min at 4°C. The absorbance was read at $OD_{540\ nm}$ and the % hemolysis was calculated using the formula

$$\% \text{ Hemolysis} = \frac{(OD_{540\ nm} \text{ of sample}) - (OD_{540\ nm} \text{ of blank})}{(OD_{540\ nm} \text{ of positive control})} \times 100$$

2.10.3. Demonstration of surface association of sphingomyelinase(s) in *Leptospira interrogans* serovar Lai.

2.10.3.1. Confocal microscopy and immunofluorescence studies (Cullen *et al*., 2005)

Preparation of reagents

1) Neutralized anti-Sph antibodies: 100 µL of anti-Sph antisera was incubated O/N with

25 µg of purified rSph3 protein at room temperature. This was diluted as required for the different experiments.

An immunofluorescence study to detect sphingomyelinase(s) was performed by confocal microscopy using a Leica TCS SP2 AOBS microscope. A 1:100 dilution of neutralized and normal anti-Sph antibodies were used in the test and for remaining procedure, refer methodology 2.7.10.

2.10.3.2. SDS-PAGE and western blotting analysis of OMPs

OMPs of *L. interrogans* serovar Lai grown under high- and low-iron conditions were separated by 5 – 20% gradient SDS-PAGE, electrophoretically transferred on to nitrocellulose membrane and probed with anti-Sph / leptospirosis patient's antisera. For detailed procedure, refer methodologies 2.7.6 and 2.7.7.

2.10.3.3. Purification and sequencing of the 63 kDa OMP (Yeruva *et al.*, 2006)

Purification of the 63 kDa OMP by gel elution and sequencing was done as per published procedure. The purified OMP sample was digested in-gel, S - alkylated and the tryptic peptides were separated by liquid chromatography and analyzed by tandem mass spectrometry (MS) on a Waters Q -TOF Ultima Global as described earlier (done in the lab of Dr. F. Altmann, Universitaet fuer Bodenkultur, Austria).

2.10.3.4. Immunoprecipitation of 63 and 60 kDa OMPs (Matsunaga *et al.*, 2005)

Preparation of solutions

1) Wash buffer: 10 mM Tris-HCl (pH 8.0).

2) TN buffer: 10 mM Tris-HCl containing 0.4 M NaCl (pH 8.0).

Immunoprecipitation of 63 and 60 kDa OMPs was done as described below. To 50 µg of OMP, 5 µL of anti-Sph antibodies were added and mixed gently at 4ºC O/N. 25 µL of EZview Red protein A affinity gel (Sigma, USA) was added and the mixture was placed on an orbital mixer at 4°C for 2 h. The immune complex bound to the protein A was recovered by centrifugation for 7 s, washed twice with 800 µL of TN buffer and finally washed once with 800 µL of wash buffer. The pellet was re-suspended in 60 µL of

2X sample buffer. Samples were boiled for 3 min, centrifuged for 7 sec and analyzed using 10% SDS-PAGE.

2.10.3.5. Homology modeling of TolC (Sali & Blundell, 1993; Sali *et al*., 1995)

Using the peptide sequences of the tryptic peptides, BLASTP analysis was done against the genome of *L. interrogans* serovar Lai strain Lai to identify the homologous region. The homologous sequence (Gene bank accession number LA0957) along with 2000 bases upstream was taken for the identification of regulatory elements.

Using *E. coli* TolC outer membrane efflux protein (1EK9, RCSB PDB) as template, 63 kDa OMP (Swiss Prot Q8F718) of *L. interrogans* serovar Lai was modeled using Insight II Modeler (Version 2000, Accelrys Inc.). The 3D structure of the leptospiral protein was built using modeler algorithm present in Insight II suite of programs. Sequence alignment with TolC of *E. coli* was done using pair-wise alignment algorithm of homology module in Insight II software followed by manual adjustments to decrease the number of gaps. Care was taken not to insert gaps in regions that were part of the regular secondary structural motifs.

The method differs from other homology programs in that it employs probability density functions (PDFs) as spatial restraints rather than energy. The main-chain conformation of a given residue in the model was described by restraints determined by the residues, main-chain conformation of equivalent residues in the reference protein and the local sequence similarity. The PDFs used in restraining the model structure were derived from correlations between structural features in a database of families of homologous proteins aligned on the basis of their 3D structures. These functions were used to restrain Cα-Cα distances, main-chain N-O distances, main-chain and side-chain dihedral angles and so on. The individual restraints were then assembled into a single molecular PDF (MPDF), with each PDF having a similar meaning as the energy terms in a molecular mechanics (MM) force field function. The PDFs, originally constructed from over 400 protein structures in the Protein Data Bank (PDB) were used with information from the template protein to build a final MPDF of the reference protein. The reference protein structures were used to derive spatial restraints for each of the restrained features of the model.

For the aligned residues, all their atomic coordinates were copied from template protein according to the restraints. However, for the mismatched residues only the C-α atom coordinates were copied from the template protein while the remaining atomic coordinates were constructed using internal coordinates derived from a CHARMM (CHemistry At HARvard Macromolecular Mechanics) (Brooks *et al*., 1983) topology library. This information of the coordinates along with the PDFs was used to build a final MPDF for the reference protein, followed by optimisation to get the final 3D model of the reference protein. The optimisation procedure consisted of a variable target function method with a conjugate gradient minimization scheme that was designed to find the most probable 3D structure of a protein, given its amino acid sequence and its alignment with related structures.

CHAPTER 3

EXPERIMENTAL RESULTS

3.1. Direct demonstration of HbpA expression upon iron-limitation in *Leptospira*.

3.1.1. Establishment of conditions of iron-limitation for the growth of pathogenic *Leptospira* spp.

Iron-limitation was achieved by the addition of iron chelators such as EDDA, EDDHPA, desferrioxamine and 2', 2' - dipyridyl to liquid EMJH medium. High-iron conditions were maintained by adding iron at 4 μg Fe / mL to liquid EMJH medium. Upon addition of the iron chelators, the growth was monitored regularly by dark-field microscopy. With 200 μM EDDHPA and 150 μM 2', 2' – dipyridyl, there was considerable cell death. Among the different chelators analyzed, appreciable growth was achieved with EDDA; hence all subsequent low-iron cultures were grown using EDDA as the iron chelator.

It was observed that addition of 200 μM EDDA to liquid EMJH medium at the time of inoculation resulted in visibly lower growth. Hence the protocol was standardized further by the addition of EDDA in a step-wise manner to the log phase culture as described below. Leptospires were initially grown in liquid EMJH medium at 30°C until the culture reached mid log phase. The log phase culture was scaled up gradually over a period of 10 days with the same medium and then divided equally into two, representing high-iron and low-iron conditions respectively. To the low-iron culture, equal volume of iron-free medium and 100 μM EDDA was added. After 24 – 48 h of incubation, the iron concentration was further decreased by the addition of an additional EDDA to obtain final concentration of 200 μM. To the high-iron culture, an additional 4 μg Fe / mL was added in two split doses at corresponding time periods of addition of chelators to low-iron culture. The low-iron organisms were obtained by centrifugation and re-suspended in iron-free medium that was pre-incubated overnight with 200 μM EDDA and incubated for 5½ h at 37°C before harvesting. The corresponding high-iron organisms were re-suspended in EMJH-BSA medium containing 10 μg Fe / mL for the same time interval and temperature as done for the corresponding low-iron organisms.

3.1.2. Demonstration of expression of hemin-binding protein HbpA in low-iron organisms of *Leptospira interrogans* serovar Lai

Leptospira interrogans serovar Lai organisms were grown under high- and low-iron conditions. The whole cell sonicates were resolved by 5 – 20% gradient SDS-PAGE and probed with anti-HbpA antibodies. A specific protein band of reactivity at 81 kDa was seen with anti-HbpA antibodies. The immunoblot analysis demonstrated the expression of HbpA in low-iron organisms only upon final incubation at 37ºC for 5½ h and could not be detected in organisms maintained at 30ºC (Fig. 10, lane 3). Low levels of recovery of HbpA was seen when outer membrane proteins (OMPs) were prepared by Triton X-114 method.

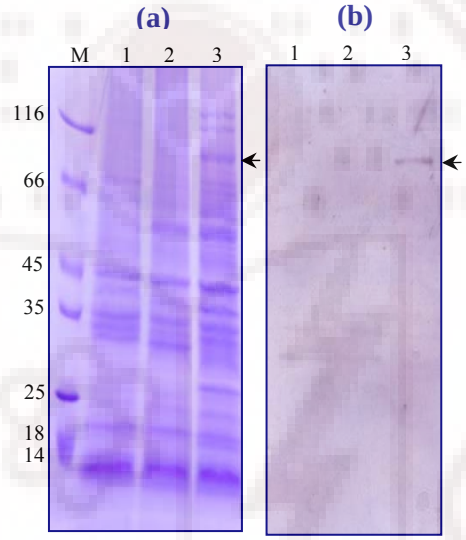


Fig. 10. Expression of HbpA in *L. interrogans* serovar Lai. The whole cell sonicate of serovar Lai was subjected to SDS-PAGE analysis and immunoblotting with rabbit anti-HbpA antibodies (Panels (a) and (b) respectively). Lanes 1, 2 and 3 represent high-iron organisms maintained at 37°C, low-iron organisms maintained at 30°C and low-iron organisms maintained at 37°C respectively in Panel (a) while the corresponding lanes in Panel (b) represent the immunoblot. Lane M is the protein molecular weight marker. The arrow indicates the 81 kDa protein band.

3.1.3. HbpA is absent in species other than *Leptospira interrogans*

Other pathogenic serovars, including *L. borgpetersenii* serovar Tarassovi strain Perepelitsin (Fig. 11a), *L. kirschneri* serovar Grippotyphosa strain Moskva V (Fig. 11b), *L. santarosai* serogroup Sarmin serovar Weaveri strain CZ390 and non-pathogenic *L. biflexa* serovar Andamana strain CH11 (Fig. 11c) did not express HbpA upon iron-limitation.

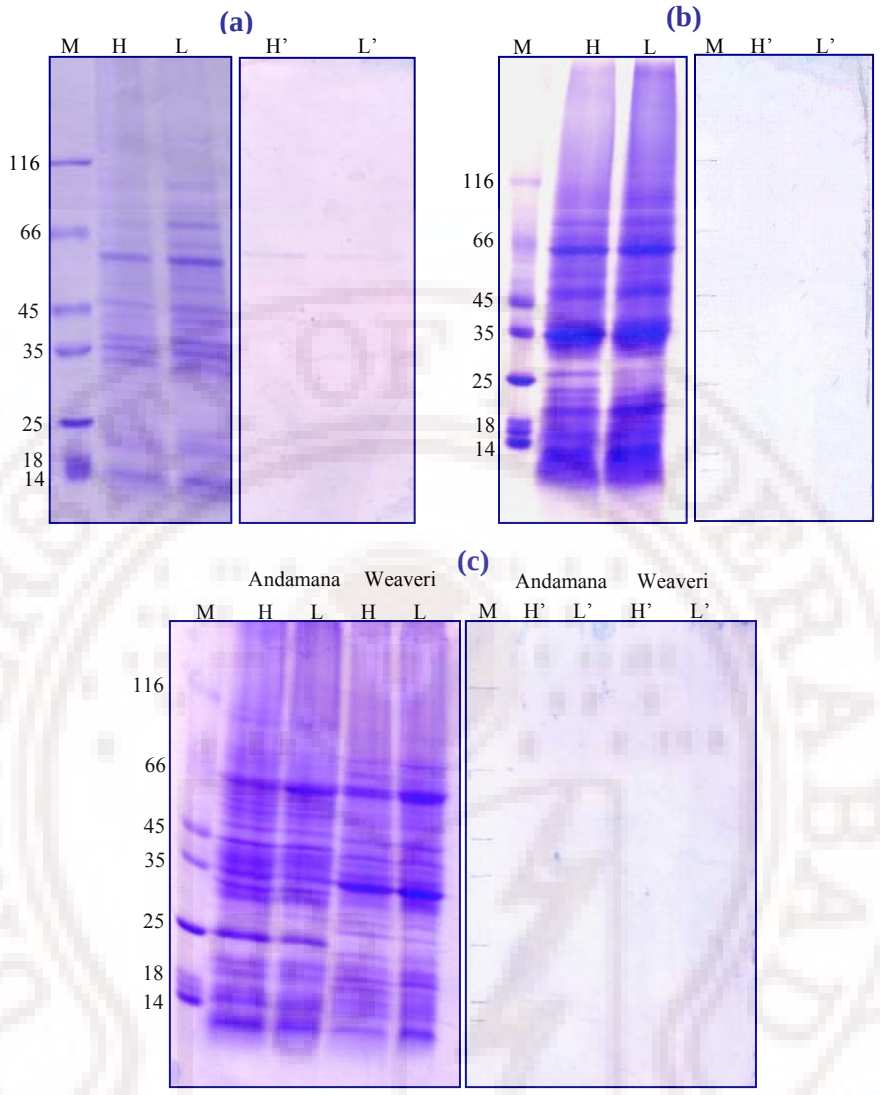


Fig. 11. HbpA is absent in leptospiral species other than *L. interrogans*. Each Panel shows the protein profile as analysed by 5 – 20% gradient SDS-PAGE and the corresponding immunoblots of whole cell sonicate of (a) *L. borgpetersenii* serovar Tarassovi strain Perepelitsin, (b) *L. kirschneri* serovar Grippotyphosa strain Moskva V and (c) *L. biflexa* serovar Andamana strain CH11 and *L. santarosai* serovar Weaveri strain CZ390 developed with rabbit anti-HbpA antibodies. Lanes H and L represent high- and low-iron organisms while the corresponding lanes (H' and L') show the immunoblot. Lane M is the protein molecular weight marker.

3.1.4. Identification of a constitutively expressed 44 kDa hemin-binding protein in *Leptospira* spp.

OMPs of high- and low-iron organisms of *L. interrogans* serovar Lai were subjected to hemin-agarose affinity binding, followed by electrophoretic separation on a 10% gel. A prominent 44 kDa hemin-binding protein was identified (Fig. 12A, Panel (b),

lane 3). This 44 kDa protein was sequenced by tandem mass spectrometry. Two distinctive peptides, ANLATYYFSTGDFEK and IGNLIGAEAILYIGYQK were obtained, which unambiguously identified the protein as LipL41 in the genome of *L. interrogans* serovar Lai. This observation was further confirmed by immuno-reactivity of the hemin-agarose purified 44 kDa protein with specific anti-LipL41 antibodies (Fig. 12A, Panel (c), lane 5). OMPs of several serovars, including the non-pathogenic *L. biflexa* serovar Patoc strain Patoc - 1, were also subjected to hemin-agarose binding (Fig. 12B, Panel (a), lanes 1 - 4). Unexpectedly, *L. biflexa* also showed the 44 kDa band along with other pathogenic serovars, which also reacted specifically with anti-LipL41 antibodies (Fig. 12B, Panel (b), lane 6).

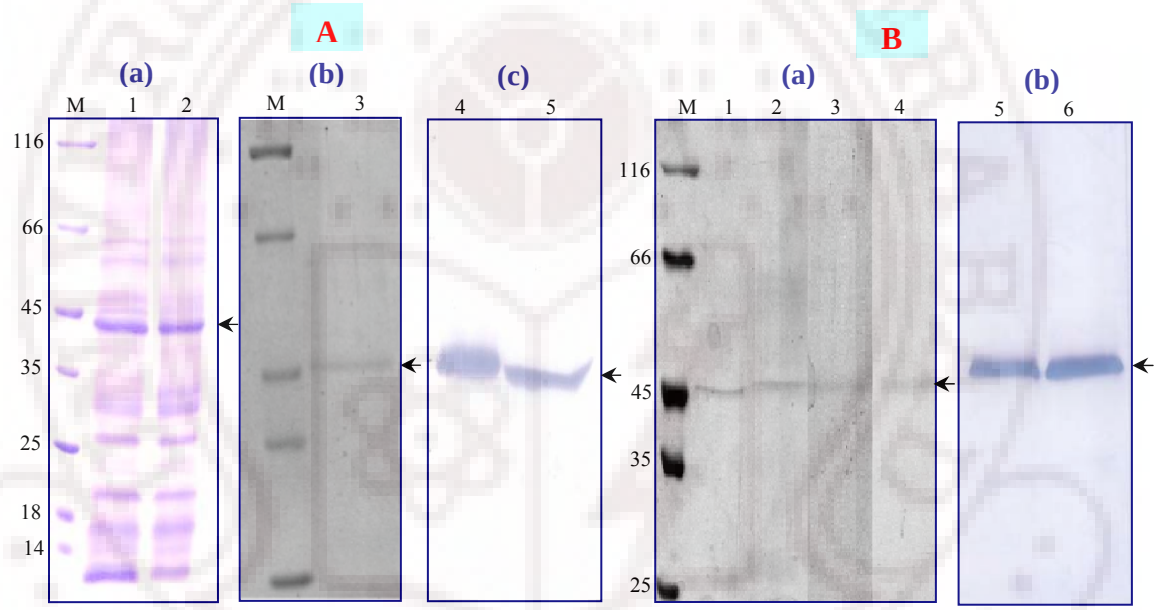


Fig. 12. Identification of a constitutively expressed outer membrane hemin-binding protein as LipL41.

(A) Observations in *L. interrogans* serovar Lai. Panel (a) represents the SDS-PAGE profile of OMPs of high- (lane 1) and low-iron organisms (lane 2); Panel (b) shows the 44 kDa band (lane 3) eluted from hemin-agarose beads. Panel (c) represents the immunoblot of OMPs from high-iron cells and the 44 kDa purified protein (lane 4 & 5 respectively) developed with anti-LipL41 antibodies.

(B) Detection of the 44 kDa protein from other leptospiral species. Panel (a) shows SDS-PAGE analysis with lanes 1 to 4 representing 44 kDa band eluted from hemin-agarose beads from OMPs of high-iron organisms of *L. borgpetersenii* serovar Ballum strain MUS 127, *L. biflexa* serovar Patoc strain Patoc I, *L. interrogans* serovar Pomona strain Pomona, and *L. kirschneri* serovar Grippotyphosa strain Moskva V respectively. Panel (b) represents the immunoblot developed with anti-LipL41 antibodies. Lanes 5 & 6 represents OMPs from high-iron cells of serovars Lai and Patoc respectively. Lane M represents protein molecular weight marker. The arrow indicates the 44 kDa protein band.

3.1.5. Demonstration of a 42 kDa transferrin-binding protein (Tbp) in *Leptospira kirschneri* serovar Grippotyphosa

OMPs from *L. kirschneri* serovar Grippotyphosa strain Moskva V grown under high- and low-iron conditions were separated on 5 – 20% gradient polyacrylamide gel and transferred onto nitrocellulose membrane. The nitrocellulose membrane was incubated with human transferrin that was detected using goat anti-human transferrin antibodies. A protein band of approximate molecular mass of 42 kDa was identified in the OMPs of low-iron organisms (Fig. 13).

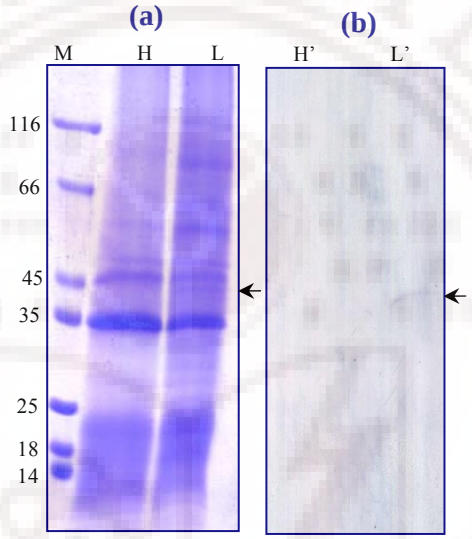


Fig. 13. Identification of a 42 kDa outer membrane transferrin-binding protein (Tbp) in *L. kirschneri* serovar Grippotyphosa. Panel (a) shows the outer membrane protein profile of high- (lane H) and low-iron organisms (lane L) as analysed by SDS-PAGE. Panel (b) represents the immunoblot of an identical set of OMPs that was incubated with human transferrin, followed by the detection of the bound transferrin with anti-human transferrin antibodies. The arrow indicates the 42 kDa transferrin-binding protein.

3.1.6. Studies on the HbpA of *Leptospira interrogans* serovar Lai

3.1.6.1. Demonstration of cell surface expression of HbpA by confocal microscopy

Immunofluorescence studies using anti-HbpA antibodies demonstrated that low-iron organisms expressed significant HbpA (Fig. 14, A1). Negligible fluorescence was observed with high-iron organisms (Fig. 14, B1). This was evident in the FITC images and in the composite images (Fig. 14, A3 & B3), with the counter stain propidium iodide showing the equivalent numbers of low- and high-iron organisms used in the study (Fig. 14, A2 & B2). Immuno-detection of the organisms using anti-LipL41 antibodies showing

equal fluorescence in both high- and low-iron organisms served as control (Fig. 14, C1 & C2).

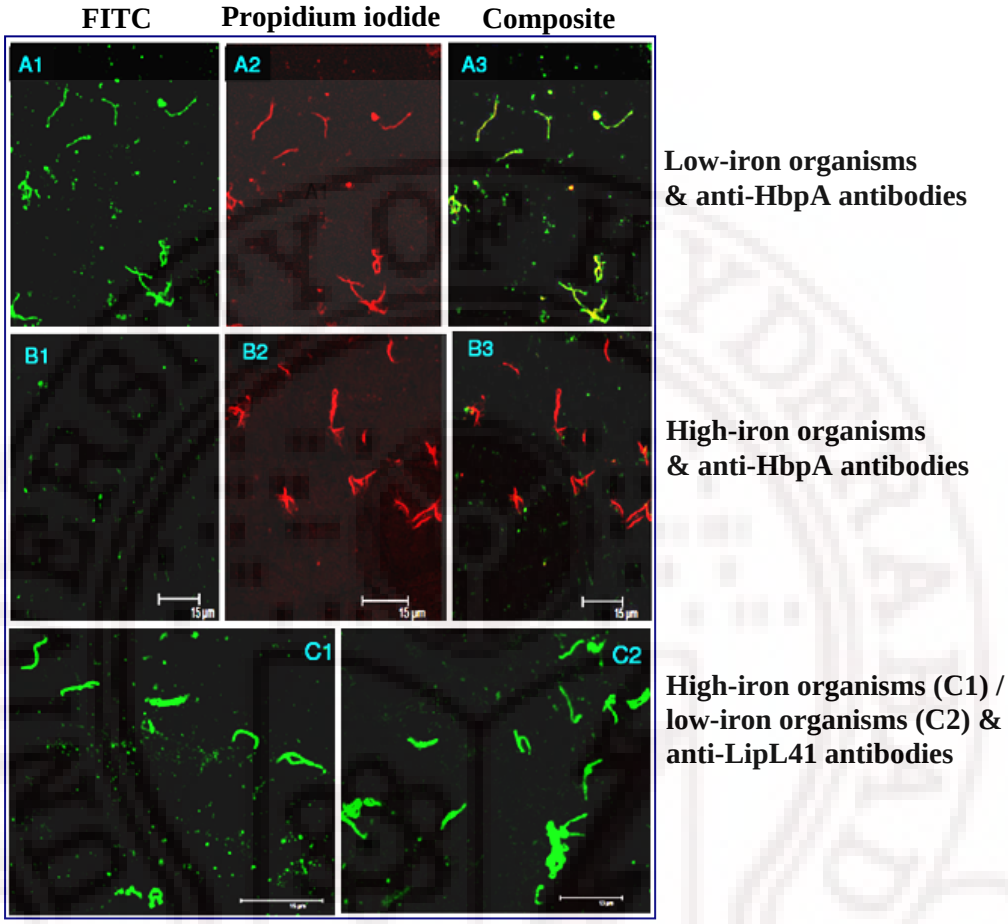


Fig. 14. Surface expression of iron-regulated HbpA in serovar Lai. Smears of low-iron (A1 to A3) and high-iron (B1 to B3) organisms were incubated with anti-HbpA antibodies and detected using FITC-conjugated secondary antibody. The bacteria were counter stained with the DNA stain propidium iodide. The constitutive expression of LipL41 in high- and low-iron (C1 & C2) organisms from the same culture is shown as control showing equivalent numbers of organisms.

3.1.6.2. Cloning and expression of $HbpA_{55}$

The HbpA sequence was analyzed for the presence of FR (A/P) P – NPNL motif and signal peptide. A FR (A/P) P – NPNL motif was identified in C – terminus between amino acid residues 472 – 475 and 495 – 498. A signal peptide cleavage site was also identified between amino acid residues 39 – 40 at the N – terminus using SignalP 3.0 (Fig. 15). The 1449 bp region of C – terminus of *hbpA* gene retaining the FR (A/P) P – NPNL motif was cloned and expressed the $rHbpA_{55}$ protein.

The 1449 bp *hbpA* gene from *L. interrogans* serovar Lai strain Lai was PCR amplified using gene specific primers (Fig. 16a). The *hbpA* amplicon and pET-28a(+) plasmid subjected to double digestion using *NdeI* and *HindIII* (Fig. 16b) were mixed and ligated O/N (pUH-LIB1) (Fig. 16c). The ligation mix was transformed into *E. coli* DH5α and the transformants carrying the *hbpA* gene were screened by colony PCR (Fig. 16d). Recombinant plasmid from a positive clone was isolated and re-transformed into the expression host *E. coli* BL21 (DE3). Positive clones were identified by colony PCR of 1449 bp using *hbpA* specific primers (Fig. 16e).

The recombinant protein was expressed by induction with 1 mM IPTG for 2 h at 37ºC (Fig. 17b). It was obtained as insoluble fraction. The recombinant protein was purified to apparent homogeneity using Bug Buster Ni-NTA His-bind resin affinity chromatography under denaturing conditions, as evidenced by the single band of 55 kDa on SDS-PAGE (Fig. 17c, Lane 4). Anti-HbpA antibodies were raised in rabbit and shown to react significantly with $rHbpA_{55}$ (Fig. 17c, Lane 4').

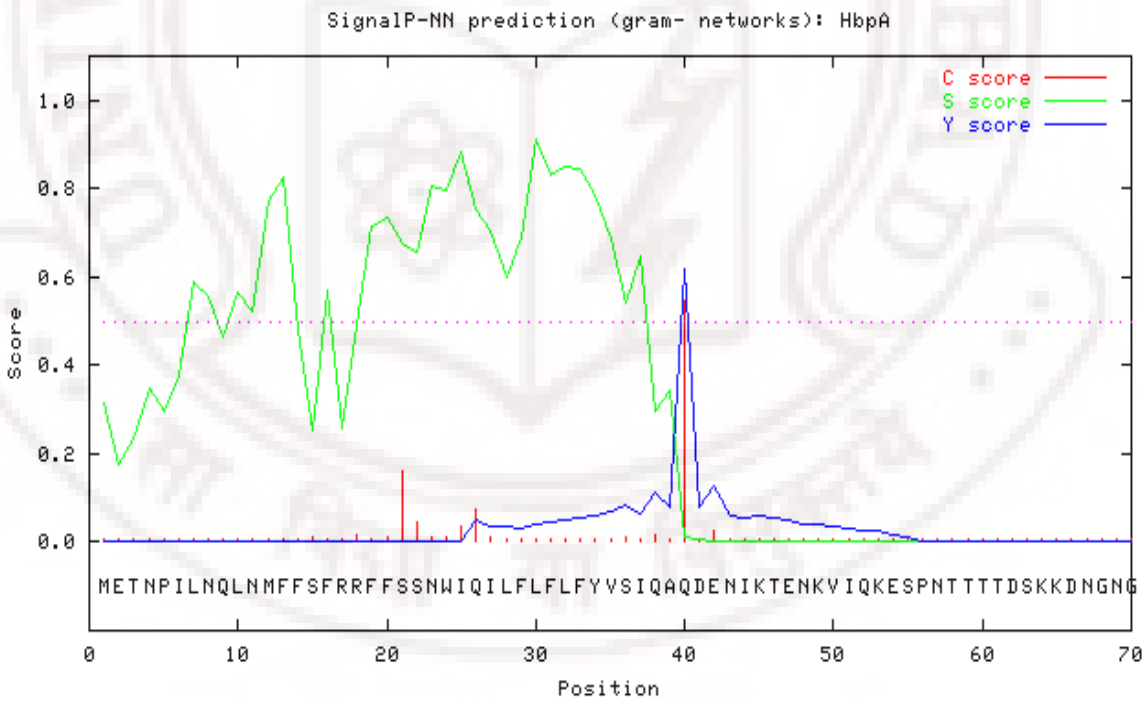


Fig. 15. Identification of signal peptide cleavage site in HbpA. A signal peptide cleavage site was detected between amino acid residues 39 – 40 (IQA – QD) at the N – terminus of HbpA using SignalP 3.0.

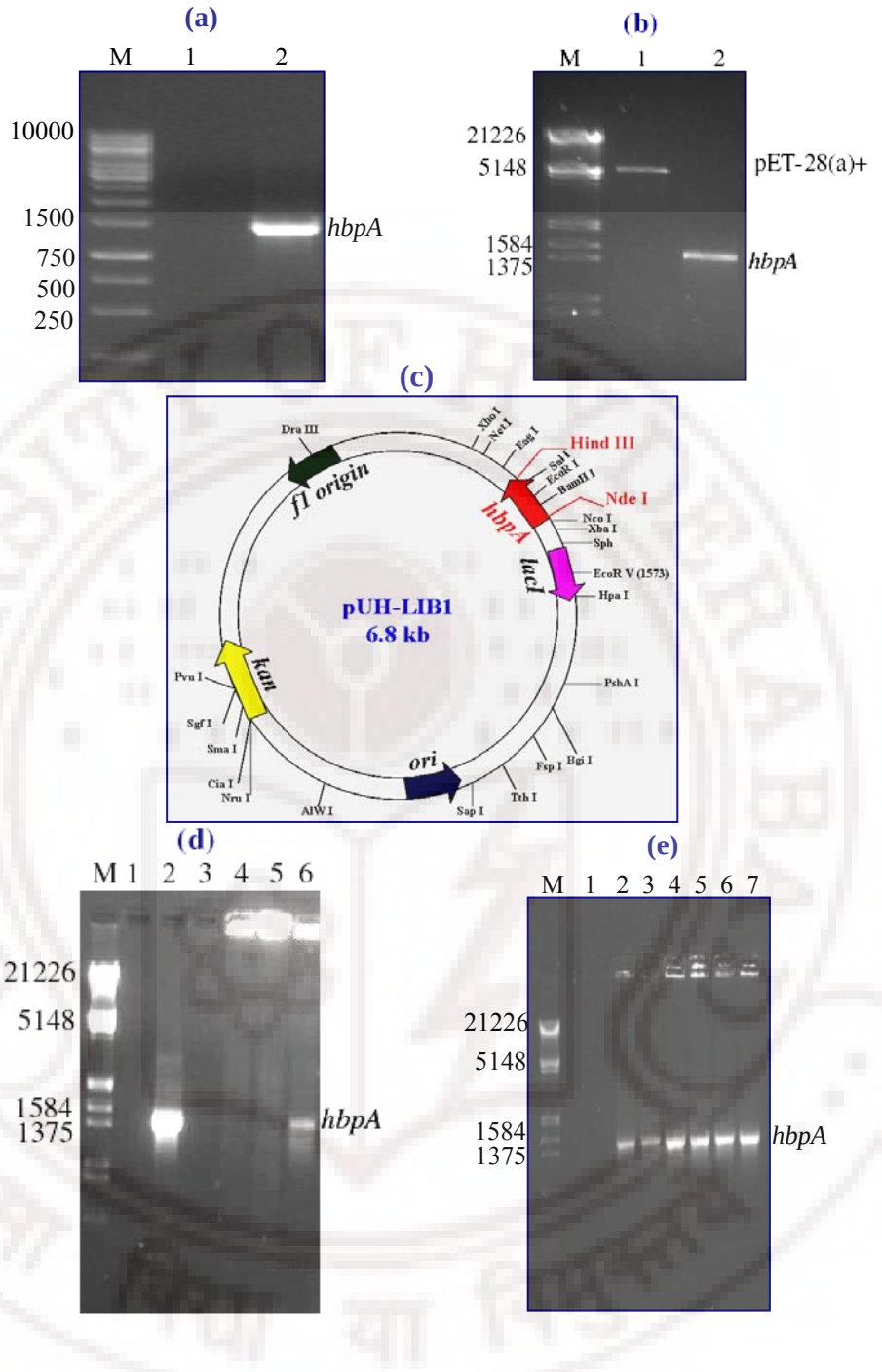


Fig. 16. Cloning of the 1449 bp *hbpA* gene fragment from *L. interrogans* serovar Lai.
Panel (a): 1449 bp *hbpA* amplicon (lane 2) amplified using *hbpA*-specific primers.
Panel (b): restriction digestion of pET 28a(+) (lane 1) and *hbpA* (lane 2).
Panel (c): map of the recombinant plasmid pUH-LIB1 with *hbpA* insert.
Panel (d): colony PCR of transformed *E. coli* DH5α transformants. The recombinant clone (lane 6) shows the 1449 bp insert as observed in the positive control (lane 2). Lane 1 is reagent blank and lanes 3 - 6 represent different colonies selected for analysis.
Panel (e): colony PCR of *E. coli* BL21 (DE3) 6 transformants (lanes 2-7) for expression studies. Lane M is molecular marker; 1 kb ladder in (a) and λ DNA (EcoRI / HindIII double digest).

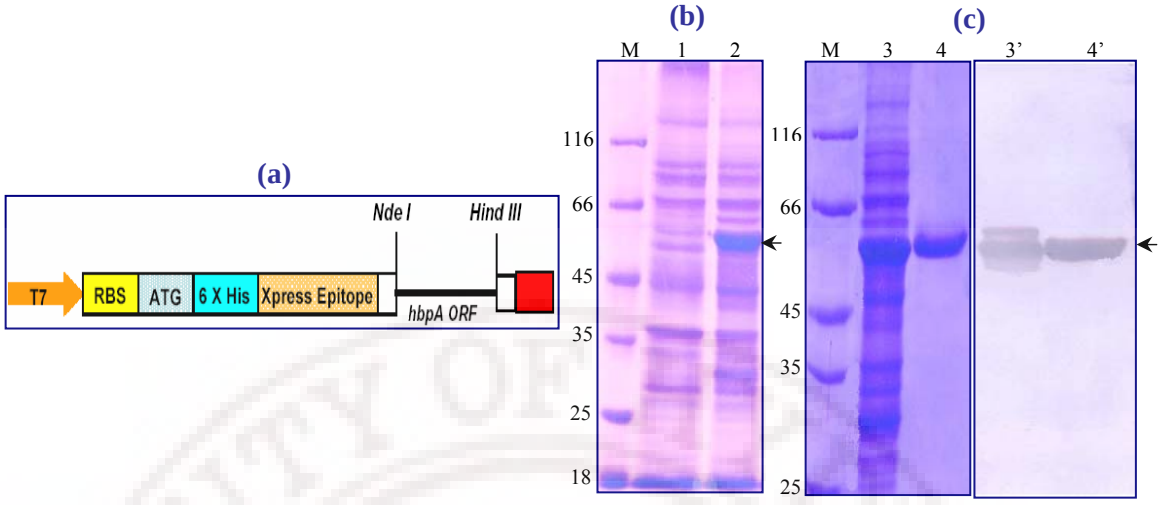


Fig. 17. Expression and purification of rHbpA$_{55}$. Panel (a): *hbpA* cassette in pUH-LIB1. Panel (b): expression of rHbpA$_{55}$; lanes 1 & 2 represent SDS-PAGE of *E. coli* lysate of un-induced and induced sample harvested 2 h after upon IPTG induction. Panel (c): SDS-PAGE of Ni-NTA column-purified rHbpA$_{55}$ (lane 4) from the whole cell lysate (lane 3). The corresponding lanes (3' & 4') represent the corresponding immunoblot developed with anti-HbpA antibodies. Lane M represents the protein molecular weight marker and the arrow indicates rHbpA$_{55}$ protein.

3.1.6.3. Hemin-binding activity of rHbpA$_{55}$

The un-induced and induced whole cell sonicates of *E. coli* BL21 (DE3) were subjected to hemin-agarose affinity chromatography, followed by electrophoretic separation on a 10% gel. The 55 kDa rHbpA$_{55}$ protein bound to hemin-agarose beads significantly (Fig. 18, lane 2). This was applied to the purification of HbpA by hemin-agarose affinity chromatography.

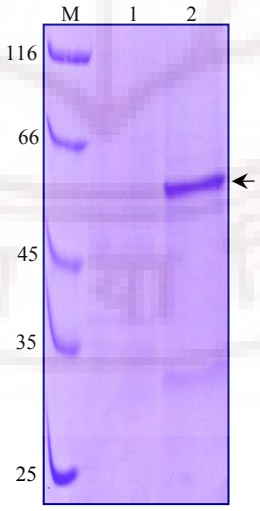


Fig. 18. Affinity binding of rHbpA$_{55}$ to hemin-agarose. Lanes 1 & 2 represents *E. coli* BL21 (DE3) lysates of un-induced and IPTG induced cells after hemin-agarose binding. Lane M represents protein molecular weight marker and the arrow indicates rHbpA$_{55}$.

3.2. Evaluation of the diagnostic potential of HbpA

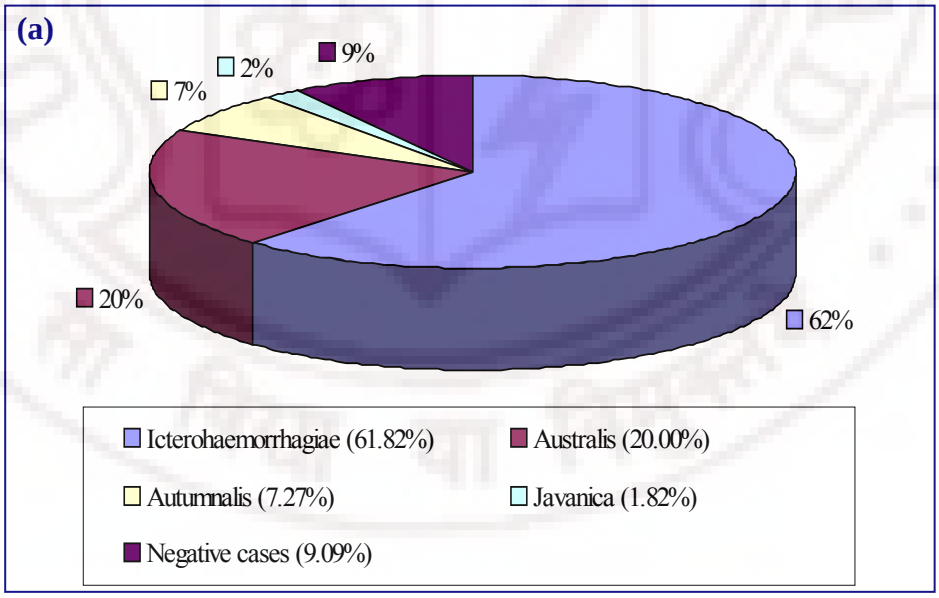
3.2.1. Immunological methods

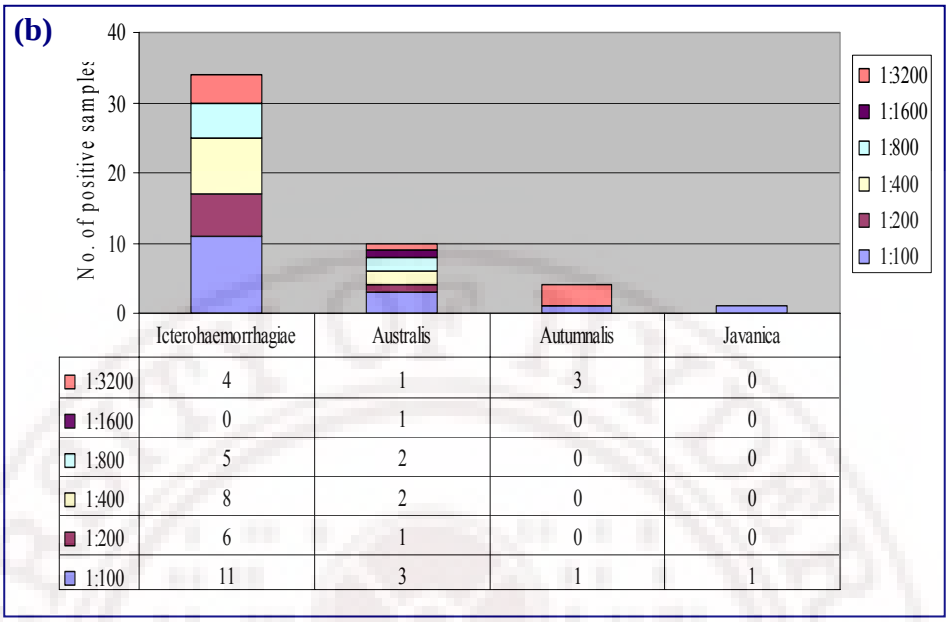
3.2.1.1. Screening of serum samples by Microscopic agglutination test (MAT)

a. Serum samples from human patients with leptospirosis

Serum samples from 55 human subjects suspected of leptospirosis were screened using LeptoTek Dri-Dot, IgM ELISA and MAT. Among 55 sera tested by LeptoTek Dri-Dot, 52 of them were positive. IgM ELISA was performed on 32 samples and 28 of them were positive. Of the 55 serum samples screened by MAT, 50 were positive; the predominant serogroup was Icterohaemorrhagiae (34/55, 62%) (Fig. 19a). Among the remaining cases, 11 had antibodies against Australis (9%), 4 against Autumnalis (7%) and 1 against Javanica (2%). Fig. 19b represents the significant titres of antibody against the different serovars, with 16% of the positive cases showing a titre of 1:3200.

Comparative analysis of LeptoTek Dri-Dot with MAT revealed relatively high sensitivity (96%) than IgM ELISA with a positive predictive value of 87%. The sensitivity of IgM ELISA as compared to MAT was found to be lower (86.7%) (Table 10).





	Icterohaemorrhagiae	Australis	Autumnalis	Javanica
1:3200	4	1	3	0
1:1600	0	1	0	0
1:800	5	2	0	0
1:400	8	2	0	0
1:200	6	1	0	0
1:100	11	3	1	1

Fig. 19. (a) Serotyping of serum samples of patients with leptospirosis by MAT and (b) antibody titres among the different serovars as analyzed by MAT. Serum samples with end point titre ≥ 1:100 were considered as positive.

Table 10. Comparative analysis of LeptoTek Dri-Dot and IgM ELISA with MAT.

Test	LeptoTek Dri-Dot		Total	Test	IgM ELISA		Total
	(+)	(-)			(+)	(-)	
MAT (+)	48	2	50	MAT (+)	26	4	30
MAT (-)	4	1	5	MAT (-)	2	0	2
Total	52	3	55	Total	28	4	32

- Sensitivity of LeptoTek Dri-Dot as compared to MAT was 96% (48/50) and of IgM ELISA as compared to MAT was found to be 86.7% (26/30).

b. Bovine serum samples

A total of 30 bovine serum samples were randomly selected from a collection of serum samples obtained from three villages in Mahaboobnagar district. MAT analysis on these sera revealed that the serovar Pomona (33%) was the predominant serovar prevalent in the region, followed by Ballum (7%), Australis (7%), Icterohaemorrhagiae (7%), Grippotyphosa (3%) and Tarassovi (3%) (Fig. 20a). Fig. 20b represents the significant

titres of antibody against the different serovars, with 22.22 % of the positive cases showing a titre of 1:3200.

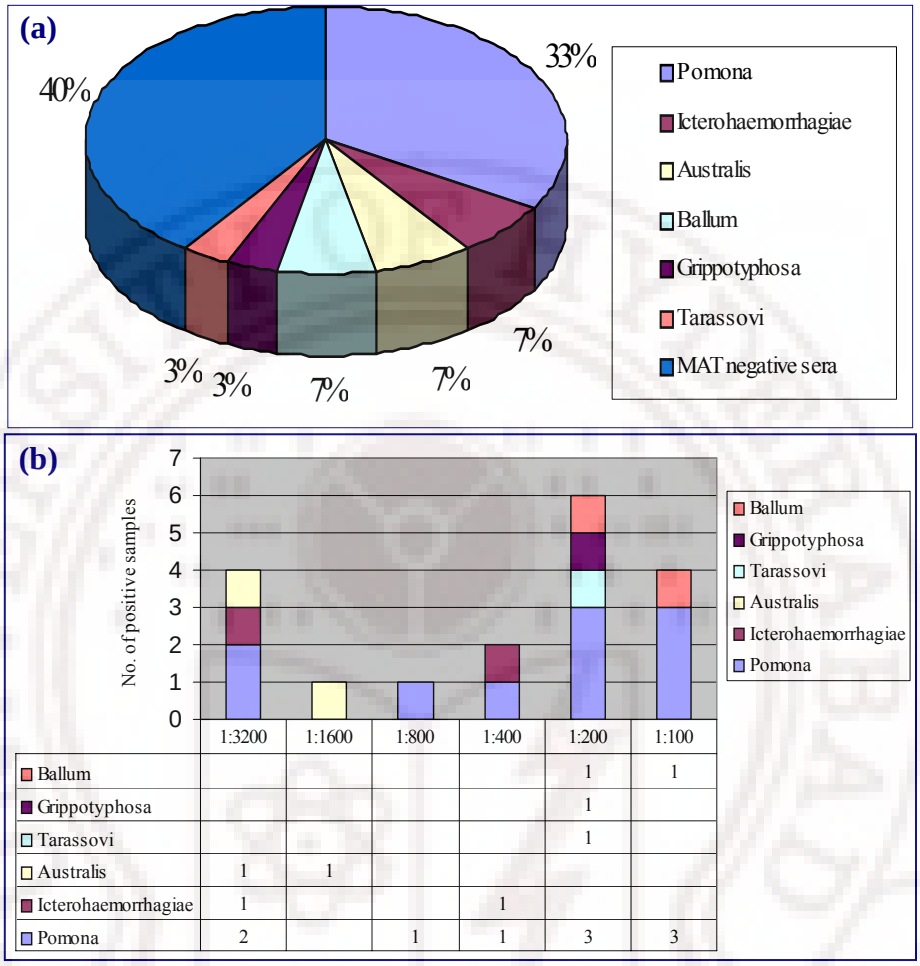


Fig. 20. (a) Serotyping of bovine serum samples by MAT and (b) antibody titres among the different serovars as analyzed by MAT. Serum samples with end point titre ≥ 1:100 were considered as positive.

3.2.1.2. Dot blot analysis: detection of anti-HbpA antibodies in serum samples of human patients with leptospirosis

Thirty five MAT positive serum samples from human patients with leptospirosis (see results 3.2.1.1a) were used for immuno-detection of antibodies against HbpA by dot blot analysis. Based on MAT data, the serum samples were grouped as GI to GV, the latter representing control serum from healthy individuals. Serum samples showing high titres of antibodies against serovar Lai were grouped as GI. Serum samples representing mixed infection, with high levels of antibodies against serovar Lai and relatively lower

levels of agglutinating antibodies against other serovars were grouped as GII. GIII included serum samples containing antibodies against *L. interrogans* serogroups Australis / Autumnalis and GIV included two samples with low levels of antibodies against serovar Lai. Thirty-three of the 35 samples were positive for serovar Lai by MAT.

Fig. 21 shows the densitometric scan of the blots. High levels of anti-HbpA antibodies (median value >27 arbitrary densitometric units) were seen in GI, GII and GIII and were statistically significant when compared to the control group GV, with P values <0.008 (Fig. 21, Table 11). The group GIV, representing only two samples also showed relatively high levels of anti-HbpA antibodies that were not statistically significant.

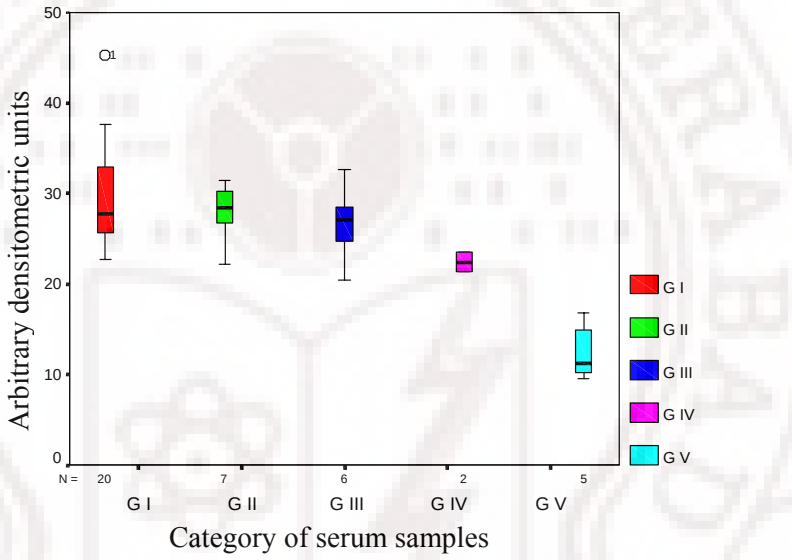


Fig. 21. Detection of anti-HbpA antibodies in serum samples from patients with leptospirosis by dot blot analysis. Figure shows the densitometric scan data of the dot blot analysis done with serum samples GI to GV. N represents the total number of samples analyzed in each group.

Table 11. Statistical analysis of densitometric scan data of dot blot assay.

S. no	Serum samples	Median value	P – value
1	Group I (n = 20)	27.763*	0.0008
2	Group II (n = 7)	28.391*	0.0058
3	Group III (n = 6)	27.102*	0.0081
4	Group IV (n = 2)	22.438	0.0841
5	Group V (n = 5)	11.240	

3.2.1.3. Sandwich ELISA for detection of anti-HbpA antibodies in serum samples

a. Purification of anti-HbpA antibodies

Anti-HbpA antibodies were subjected to immobilized *E. coli* lysate column for selective removal of contaminating anti-*E. coli* antibodies and different fractions were collected. Absorbance of the fractions was measured at $OD_{280\ nm}$ (Fig. 22) and fractions with maximum absorbance were pooled and used for further studies.

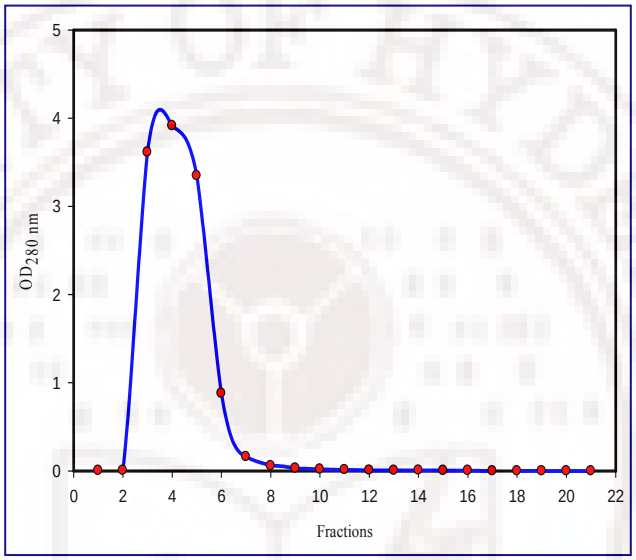


Fig. 22. Purification of anti-HbpA antibodies. Anti-HbpA antibodies were passed through commercial immobilized *E. coli* lysate column and the fractions with maximal $OD_{280\ nm}$ were pooled.

b. Cross-reactivity of anti-HbpA antibodies

The anti-HbpA antibodies were then tested for cross-reactivity by direct ELISA with whole cell sonicates of commonly infected human bacterial pathogens such as *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*. The reactivity was notably low with these human bacterial pathogens, when compared to whole cell sonicates of serovar Lai and purified $rHbpA_{55}$ (Fig. 23). When whole cell sonicates of serovar Lai were compared, the reactivity was more with low-iron organisms compared to organisms grown under high-iron condition.

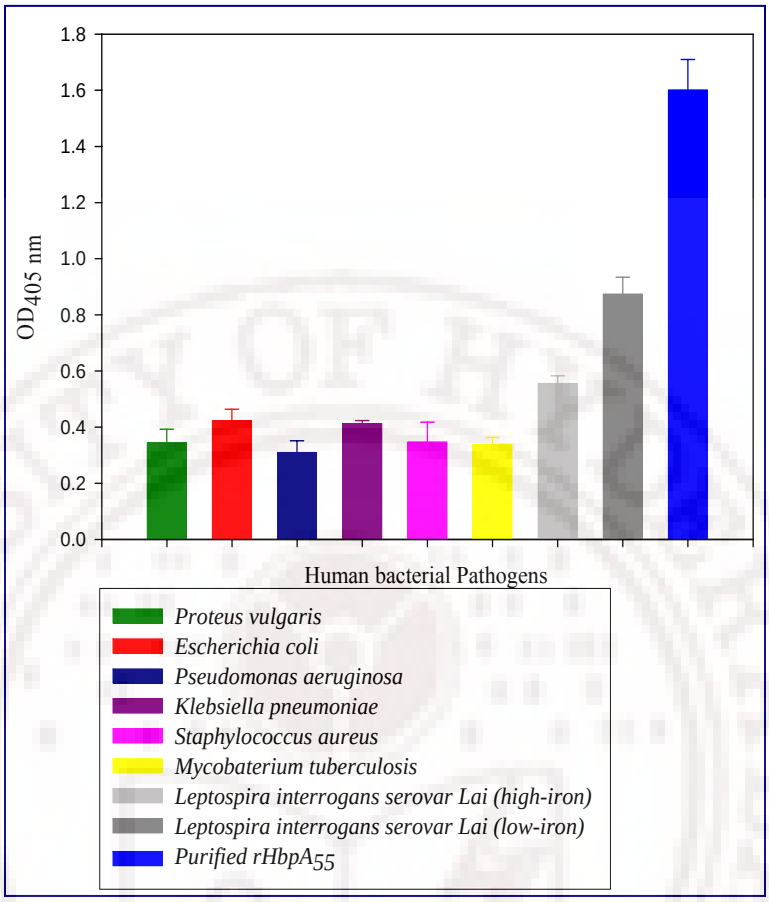


Fig. 23. ELISA: assay of the cross-reactivity of anti-HbpA antibodies with other bacterial pathogens. ELISA was performed using whole cell sonicates of bacterial pathogens against anti-HbpA antibodies and absorbance was measured at $OD_{405\ nm}$. The vertical bars represent the standard deviation of the mean from three independent experiments.

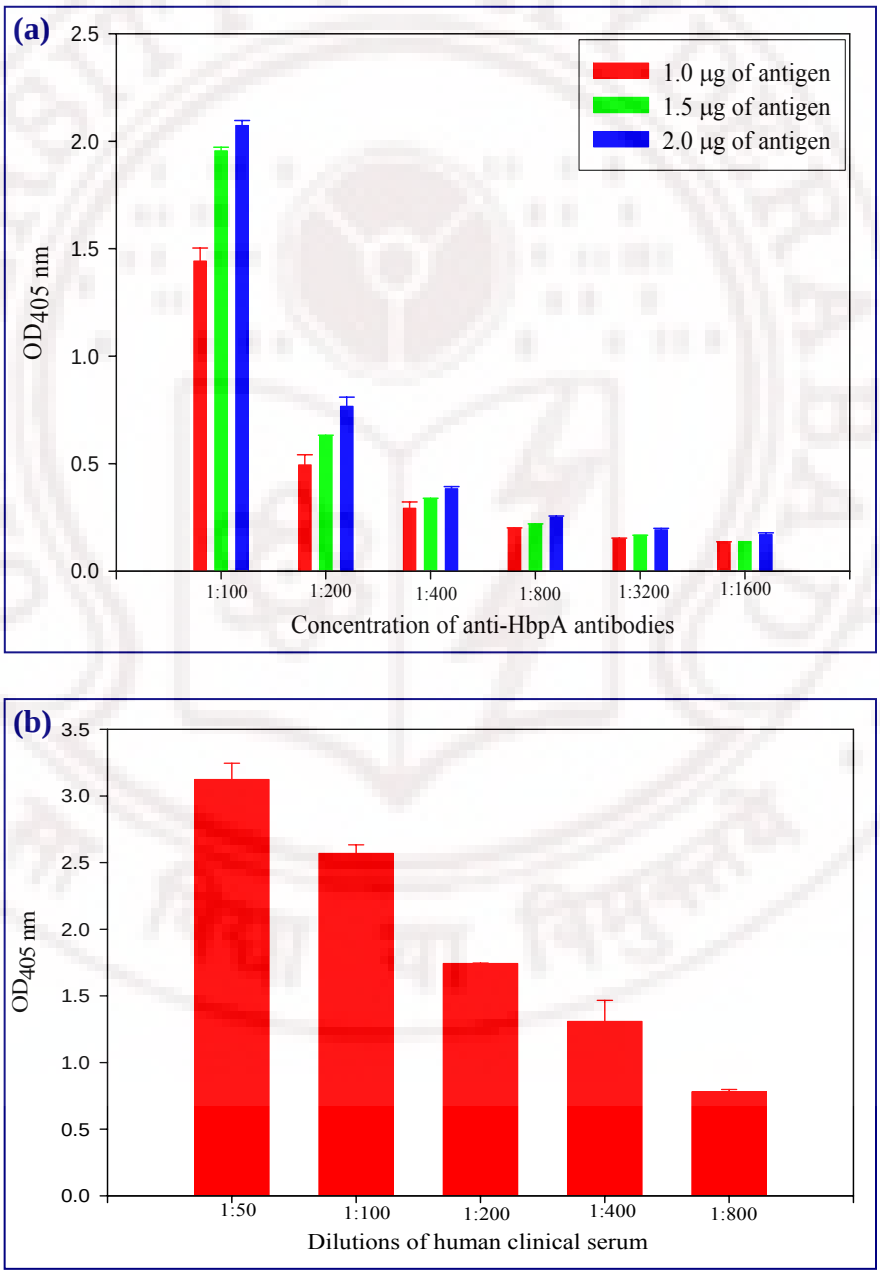
c. Standardization of sandwich ELISA

The optimum concentrations of antigen and antibodies to be used in ELISA were standardized by checkerboard titration in direct ELISA. The $rHbpA_{55}$ antigen concentrations ranging from 1.0 to 2.0 µg were tested against anti-HbpA antibodies in a direct ELISA assay. With increase in dilution of anti-HbpA antibodies, there was proportionate decrease in absorbance ($OD_{405\ nm}$). No significant difference in the absorbance was noted with 1.5 and 2.0 µg antigen (Fig. 24a). 1.0 µg of antigen was optimized for further screening of serum samples.

Using 1.0 µg of $rHbpA_{55}$ antigen, varying concentrations of serum sample from a patient with leptospirosis was used in direct ELISA (Fig. 24b). 1:400 of serum was

selected for further standardization of sandwich ELISA as optimum absorbance was obtained at this dilution.

Sandwich ELISA was standardized by coating 1:25, 1:50 and 1:100 dilutions of anti-HbpA antibodies and 1 µg antigen was tested against 1:400 dilution of serum sample from a patient with leptospirosis (Fig. 24c). The 1:50 dilution of anti-HbpA antibodies was selected as coating antibody and 1 µg concentration of antigen was selected for further assays, as these concentrations showed optimum absorbance.



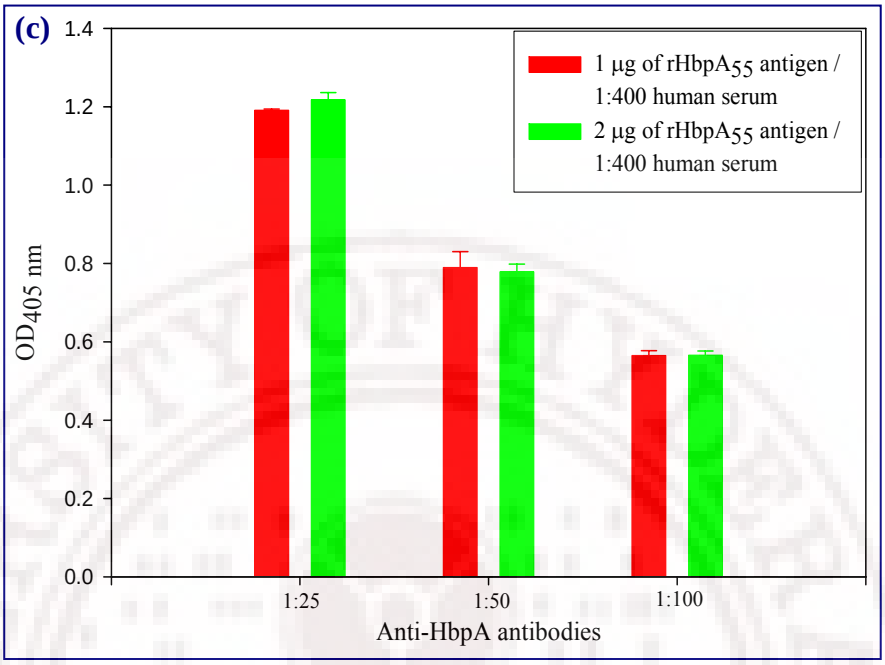


Fig. 24. Optimization of antigen and antibody concentrations for sandwich ELISA.
Panel (a): checkerboard titration of $rHbpA_{55}$ antigen vs varying dilution of anti-HbpA antibodies.
Panel (b): direct ELISA to show the immunoreactivity of 1 µg of $rHbpA_{55}$ antigen with varying dilutions of serum from a patient with leptospirosis.
Panel (c): sandwich ELISA showing the optimization of concentration of coating antibody and antigen. Human serum was diluted 1:400 and the $OD_{405\ nm}$ was recorded.
The vertical bars represent the standard deviation of the mean from three independent experiments.

d. Determination of cut-off value

For human serum samples, the cut-off value of the sandwich ELISA was calculated as 0.4497 by taking mean $OD_{405\ nm}$ plus double the standard deviation of means of five healthy controls. Similarly, the cut-off value for bovine serum samples was calculated as 0.2656.

e. Screening of serum samples from human patients with leptospirosis

Among 45 serum sample screened, 36 (80%) were positive (Fig. 25). The sensitivity, specificity, accuracy and positive predictive value of the ELISA as compared to MAT were 87.17%, 81.81%, 86.00% and 94.44% respectively (Table 12).

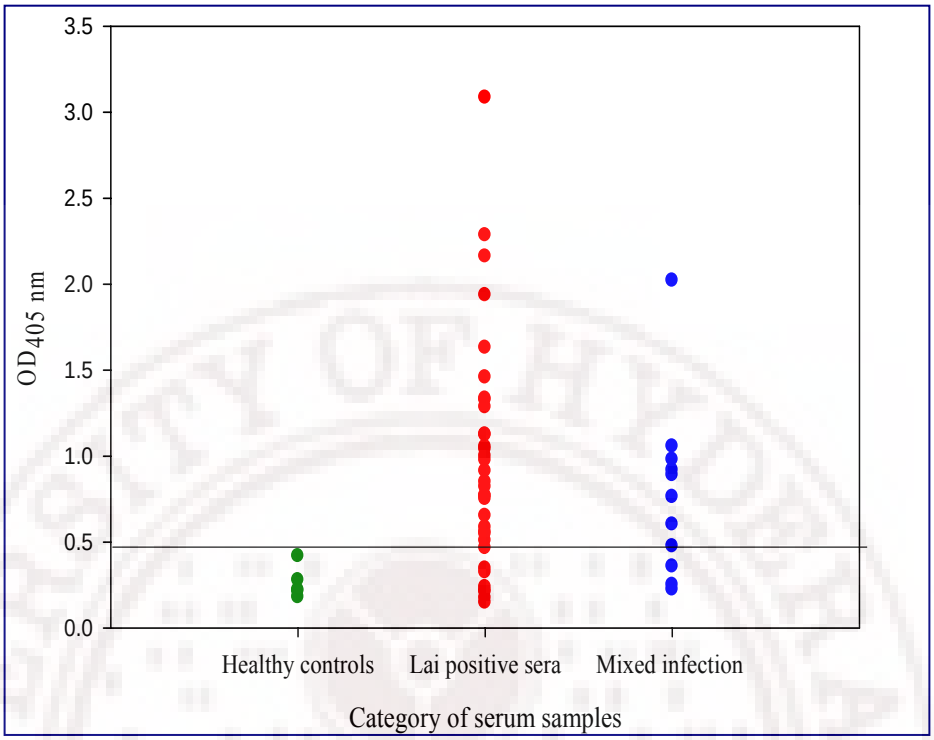


Fig. 25. Screening of serum samples from patients with leptospirosis by sandwich ELISA. 45 MAT-positive serum samples from patients were analysed by sandwich ELSIA. The majority of samples tested positive for *L. interrogans* serovar Lai in the MAT assay and were grouped as Lai positive; the second group represented mixed infection and the third group comprised of 5 healthy controls. The absorbance is the mean of triplicate values. The horizontal line indicates the cut-off value (0.4467).

Table 12. Diagnostic potential of HbpA as compared to MAT

Test	MAT		Total	Features
	(+)	(-)		
ELISA (+)	34	2	36	Positivity = 80.00%
ELISA (-)	5	9	14	Sensitivity = 87.17%
Total	39	11	50	Specificity = 81.81%

f. Screening of bovine serum samples

Among the 50 serum samples screened, 39 (78%) were positive (Fig. 26). The specificity, sensitivity, accuracy and positive predictive value of the ELISA as compared to MAT were 78.95%, 16.67%, 64.00% and 75.00% respectively (Table 13).

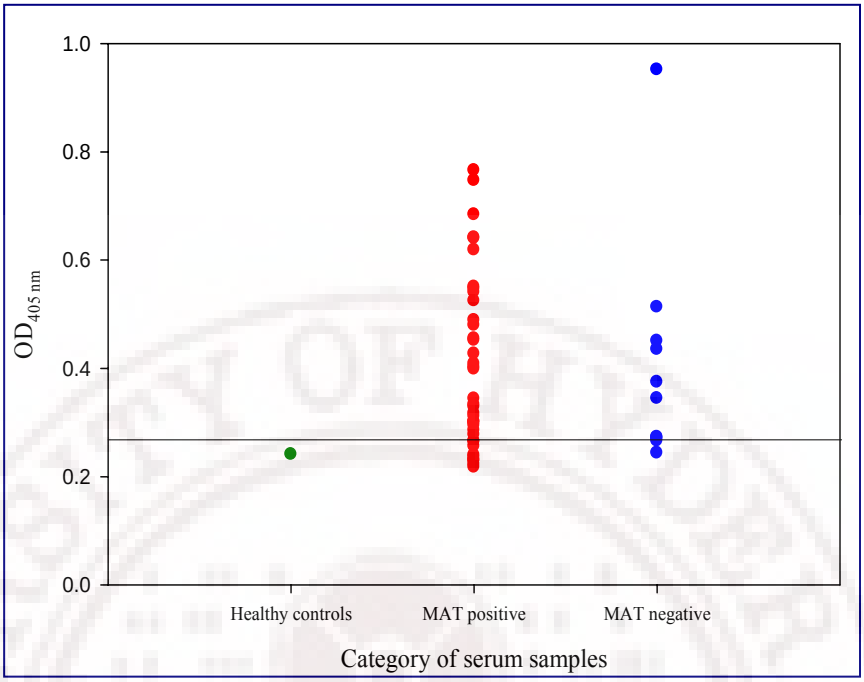


Fig. 26. Screening of bovine serum samples by sandwich ELISA. Test was performed in triplicates using 50 bovine serum samples that were previously screened by MAT. The cut-off value was calculated as 0.2656 by employing healthy controls. The absorbance is the mean of triplicate values.

Table 13. Usefulness of HbpA as a diagnostic antigen in animals.

Test	MAT		Total	Features
	(+)	(-)		
ELISA (+)	30	10	40	Positivity = 78.00%
ELISA (-)	8	2	10	Sensitivity = 78.95%
Total	38	12	50	Specificity = 16.67%

- HbpA detection did not prove useful in screening bovine serum samples.

3.2.2. Molecular methods

3.2.2.1. PCR analysis using reference strains of *Leptospira* spp.

PCR assay was performed using DNA from 20 reference strains of *Leptospira* spp. (Fig. 27). Among the pathogenic leptospires, the 2148 bp full-length *hbpA* gene was identified in the following serovars such as *L. interrogans* serovar Canicola strain HU IV (lane 2), serovar Hardjo strain Hardjoprajitno (lane 4), serovar Hebdomadis strain Hebdomadis (lane 6), serogroup Autumnalis serovar Rachmati strain Rachmat (lane 10),

serovar Australis strain Ballico (lane 11), serovar Lai strain Lai (lane 12), serovar Icterohaemorrhagiae strain RGA (lane 13), serovar Pomona strain Pomona (lane 17) and serovar Pyrogenes strain Salinem (lane 18). A smaller size PCR product was seen in *L. borgpetersenii* serovar Tarassovi (lane 6) and a slightly larger product was seen in *L. interrogans* serovar Autumnalis (lane 8). The *hbpA* gene was absent in the non-pathogenic serovars *L. meyeri* serovar Ranarum (lane 19) and *L. biflexa* serovar Patoc (lane 20).

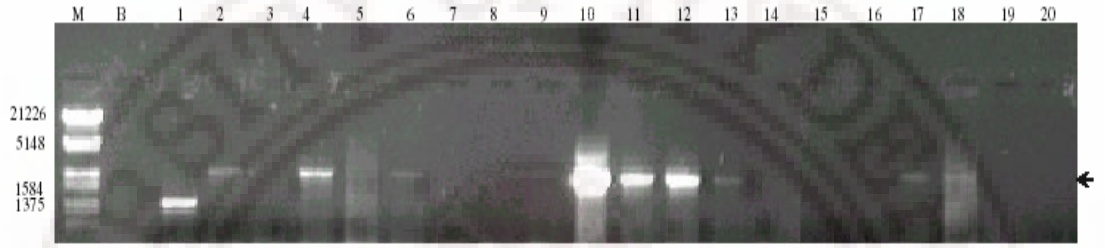


Fig. 27. PCR based detection of the full - length *hbpA* gene from leptospiral reference strains. Lanes 1 – 20 show *L. borgpetersenii* serovar Tarassovi strain Perepelitsin, *L. interrogans* serovar Canicola strain HUIV, *L. santarosai* serogroup Sarmin serovar Weaveri strain CZ390, *L. interrogans* serovar Hardjo strain Hardjoprajitno, *L. kirschneri* serovar Ratnapuira strain Wumalasena, *L. interrogans* serovar Hebdomadis strain Hebdomadis, *L. kirschneri* serovar Grippotyphosa strain Moskva V, *L. kirschneri* serovar Cynopteri strain 3522C, *L. borgpetersenii* serovar Ballum strain MUS127, *L. interrogans* serogroup Autumnalis serovar Rachmati strain Rachmat, *L. interrogans* serovar Australis strain Ballico, *L. interrogans* serovar Lai strain Lai, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. borgpetersenii* serovar Poi strain Poi, *L. noguchii* serovar Louisiana strain LSU1945, *L. noguchii* serovar Panama strain CZ214K, *L. interrogans* serovar Pomona strain Pomona, *L. interrogans* serovar Pyrogenes strain Salinem, *L. meyeri* serovar Ranarum strain ICF and *L. biflexa* serovar Patoc strain Patoc I respectively. Lanes M and B represents molecular marker (λ DNA, EcoRI / HindIII double digest) and blank (without template DNA) respectively. Arrow indicates the full-length 2148 bp PCR product.

3.2.2.2. PCR analysis of clinical isolates

Using 1449 bp *hbpA* gene specific primers, PCR analysis was performed with DNA from 91 clinical isolates of *Leptospira* spp. (Fig. 28). DNA samples were isolated from serovars belonging to *L. interrogans* [51], *L. santarosai* [9], *L. meyeri* [1], *L. kirschneri* [9], *L. noguchii* [1] and *L. borgpetersenii* [20], respectively. All the 51 isolates belonging to *L. interrogans* tested positive for *hbpA* (Table 14), with no amplification seen with the isolates belonging to the other five species.

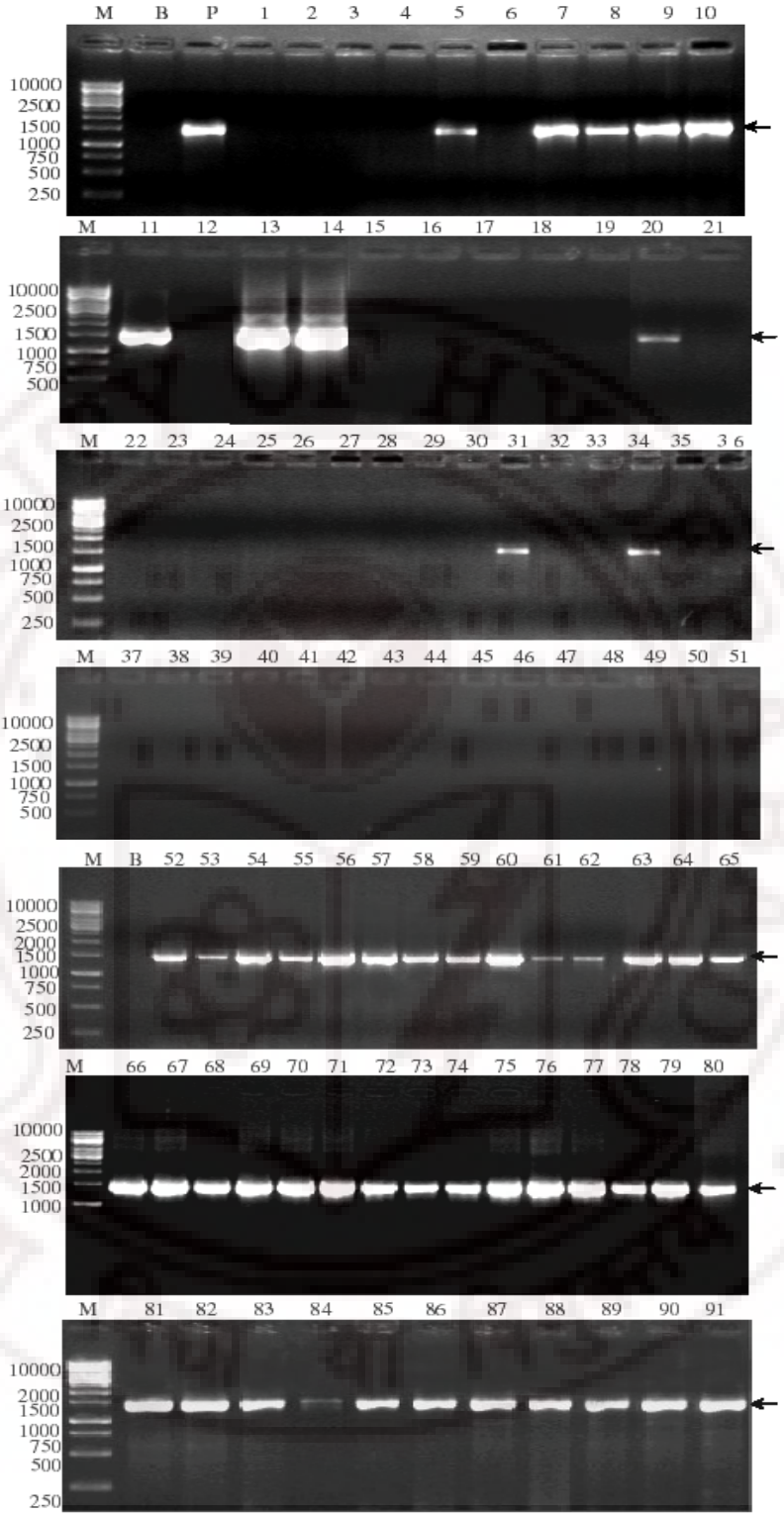


Fig. 28. *hbpA* as a diagnostic marker: PCR-based molecular analysis of clinical isolates. 91 DNA samples from clinical isolates (Table 5, Materials and Methods) were subjected to PCR and analysed after electrophoretic separation (lanes 1 – 91); samples 1 – 51 are samples from different geographical regions of the world and samples 52 – 91 are from southern parts of India. Lanes B and M represent reagent blank and 1 kb molecular marker respectively. The arrow indicates 1449 bp *hbpA* amplicon.

Table 14. Specificity of *hbpA* to *Leptospira interrogans* species: PCR analysis

Genomospecies	Number of strains tested	Strains positive for *hbpA*
L. interrogans	**51**	**51**
L. borgpetersenii	20	0
L. santarosai	9	0
L. meyeri	1	0
L. kirschneri	9	0
L. noguchii	1	0

3.3. Iron levels and expression of the sphingomyelinase(s) in *L. interrogans* serovar Lai

3.3.1. Comparative genomics: *in silico* analysis of leptospiral hemolysins

The genome analysis of *L. interrogans* serovar Lai revealed 10 hemolysin genes, including four genes (LA1027, LA1029, LA4004 & LA3050) encoding hemolysins with sphingomyelinase activity, five genes (LA3937, LA0378, LA0327, LA1650 & LA0177) encoding hemolysins with phospholipase activity and one gene (LA3540) encoding hemolysin with pore-forming activity.

The genome analysis of *L. borgpetersenii* serovar Hardjo strain JB197 revealed three genes (LBJ0291, LBJ0527 & LBJ0881) encoding hemolysins with sphingomyelinase activity and three genes (LBJ0393, LBJ2396 & LBJ1743) encoding hemolysins with phospholipase activity. In *L. borgpetersenii* serovar Hardjo strain L550, two genes (LBL0869 & LBL2785) encoding hemolysins with sphingomyelinase activity and three genes (LBL0712, LBL1962 & LBL2684) encoding hemolysins with phospholipase activity were identified.

Genome of non-pathogenic *L. biflexa* showed the presence of five putative genes (LEPBIa0082, LEPBIa0717, LEPBIa2015, LEPBIa2375 & LEPBIa2477) encoding for hemolysins with phospholipase activity and the genes encoding for hemolysins with sphingomyelinase activity were absent.

The phylogenetic analysis of hemolysin genes of serovar Lai showed that the sphingomyelinases formed a single cluster and non-sphingomyelinase hemolysins formed

another cluster (Fig. 29a). The sphH (LA3540) of serovar Lai had no corresponding ortholog in *L. borgpetersenii* strains JB197 and L550, while sph1 (LA1027), sph2 (LA1029) and sph3 (LA4004) had 2 orthologs in strain JB197 (LBJ0291 & LBJ0527) and one ortholog in strain L550 (LBL2785). The sph4 (LA3040) of serovar Lai had one ortholog each from strains JB197 (LBJ0881) and L550 (LBL0869) (Fig. 29b).

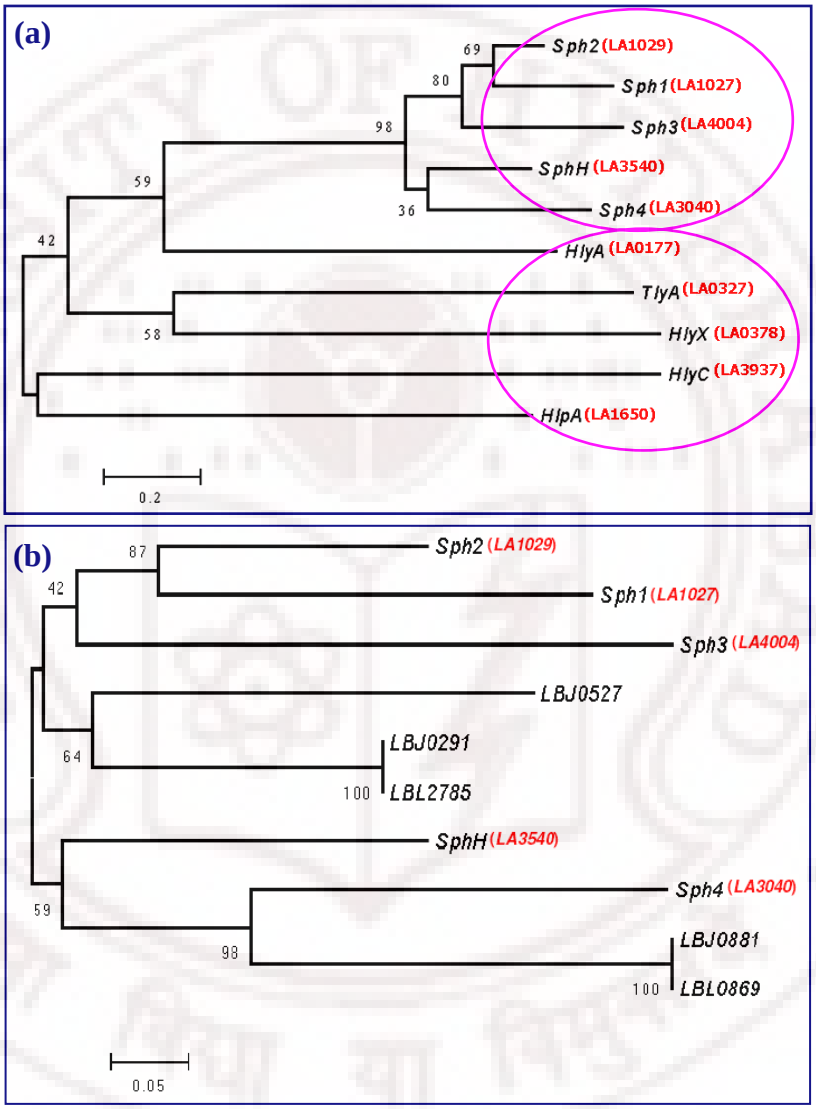


Fig. 29. Phylogenetic analysis of leptospiral hemolysins done using Clustal X and Mega 3.1 software. Hemolysins from the genome of serovar Lai (Panel a) show the sphingomyelinases as a cluster and the hemolysins as a separate cluster. The former was compared with the corresponding orthologs of *L. borgpetersenii* serovar Hardjo (Panel b). The phylogenies generated by neighborhood joining with 400 bootstrap replicates, rooted at midpoint and bootstrap values, are shown as percentages. The numbers refer to the divergence between the sequences.

3.3.2. Isolation and characterization of outer membrane vesicles (OMVs) of serovar Lai

3.3.2.1. Transmission electron microscopy (TEM)

The OMVs of the *L. interrogans* serovar Lai grown under high- and low-iron conditions were isolated from the spent growth medium by ultracentrifugation. Ultra structural analysis of OMVs was performed by TEM and visualized by 1% uranyl acetate stain (negative staining) (Fig. 30). Panel (a) shows the individual OMVs and panel (b) shows the string like release of OMVs from the surface of the organism.

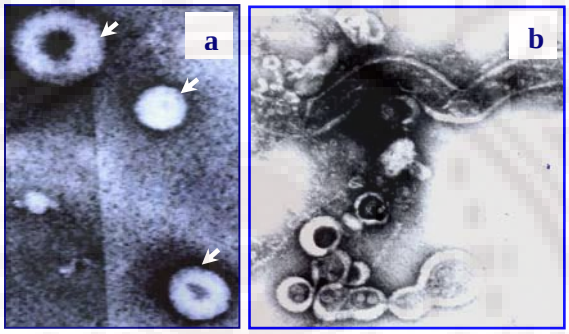


Fig. 30. Transmission electron microscopy of OMVs of *L. interrogans* serovar Lai. Panel (a) show electron micrograph of OMVs visualized by 1% uranyl acetate staining and Panel (b) show the release of the OMVs from the leptospiral cell surface as a beaded string.

3.3.2.2. Characterization of OMVs

a. OMVs of *L. interrogans* serovar Lai and *L. biflexa* serovar Andamana

Serovar Lai grown under low-iron condition produced significantly higher amount of OMVs as observed by the size of the pellet seen upon centrifugation of the spent growth medium. The non-pathogenic *L. biflexa* produced low amounts of OMVs, irrespective of the iron status of the growth medium.

The OMV proteins of the serovars Lai and Andamana, upon electrophoretic separation on 5 – 20% gradient polyacrylamide gel and immunoblotting analysis with the respective anti-Sph, anti-LipL32 and anti-HbpA antibodies revealed marked differences (Fig. 31 & 32). A major finding was the detection of a 42 kDa band with anti-Sph antibodies in the OMVs of low-iron cells of serovar Lai (Fig. 31b, lane L) that was clearly absent in the corresponding OMVs of high-iron cells of serovar Lai (Fig. 31b, lane H) and non-pathogenic serovar Andamana (Fig. 32b). Interestingly, the 81 kDa HbpA was detected in the OMVs of low-iron cells of serovar Lai (Fig. 31d, lane L). The

anti-HbpA antibodies also reacted strongly with a 29 kDa protein band that was absent in high-iron organisms. Both the 81 and 29 kDa proteins were not seen in the serovar Andamana (Fig. 32d). The blots, probed with anti-Lip32 antibodies (Fig. 31c) showed that LipL32 was relatively less upon iron-deprivation in serovar Lai. LipL32 was not detected in the serovar Andamana (Fig. 32c).

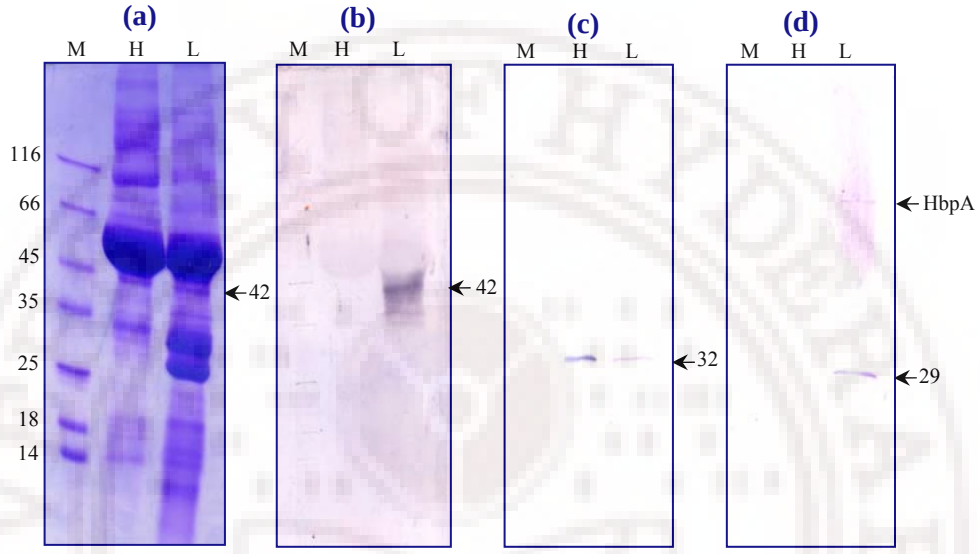


Fig. 31. Identification of iron-regulated proteins in the OMVs of serovar Lai. OMVs of high- (lane H) and low-iron (lane L) organisms, as analysed by SDS-PAGE (Panel a) and immunoreactivity with anti-Sph (Panel b), anti-LipL32 (Panel c) and anti-HbpA (Panel d) antibodies. M is the protein molecular weight marker.

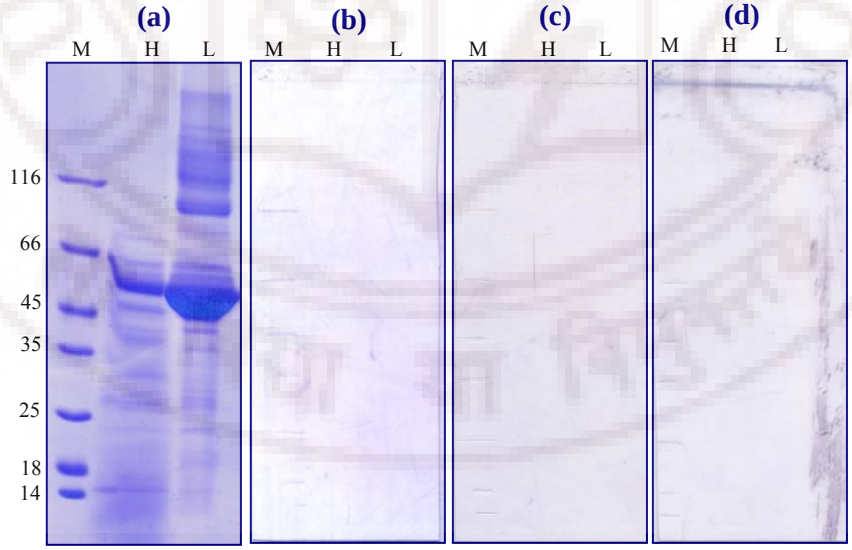


Fig. 32. Immunoreactivity of OMVs of high- and low-iron organisms of *L. biflexa* serovar Andamana with anti-Sph, anti-LipL32 and anti-HbpA antibodies. Panel (a) shows protein profile of high- (lane H) and low-iron (lane L) organisms as analysed by SDS-PAGE and Panels (b) (c) and (d) show immunoreactivity of OMVs with anti-Sph, anti-LipL32 and anti-HbpA antibodies respectively. M is the protein molecular weight marker.

b. Hemolytic activity of the OMVs of serovar Lai

The hemolytic activity of OMVs on red blood cells of sheep was assayed by measuring the absorbance of the released haemoglobin and expressed in % of positive control, with 100% lysis of the RBCs. The positive and negative controls showed 100% and zero lysis of the red blood cells, respectively. The OMVs of low-iron cells showed 2.3 times higher hemolytic activity as compared to high-iron organisms (Fig. 33). Addition of calcium chloride resulted in a further 1.5 fold increase in the hemolysis. The hemolytic activity of the OMVs of low-iron organisms decreased by 7 fold, upon pre-incubation of the OMVs with anti-Sph antibodies. Whereas 1.5 folds decrease was observed in the corresponding high-iron organisms.

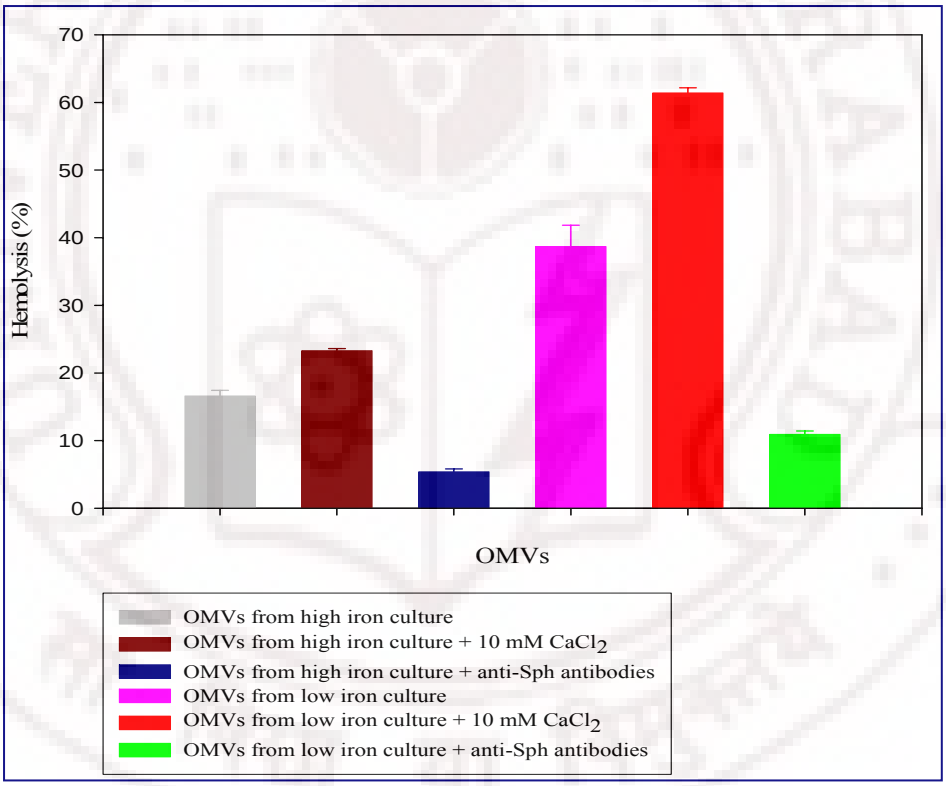


Fig. 33. Hemolytic activity of the OMVs of serovar Lai. OMVs from high- and low-iron organisms were added to sheep RBC and the resultant hemolysis was expressed as % of the complete hemolysis obtained with RBCs in double distilled water. The figure shows identical replicates of the OMVs were assessed for the hemolytic activity after the addition of 10 mM calcium chloride and pre-incubation with anti-Sph antibodies respectively. The vertical bars represent the standard deviation of the mean from three independent experiments.

3.3.3. Demonstration of surface localization of sphingomyelinases

3.3.3.1. Confocal microscopy: immunoreactivity of anti-Sph antibodies with low-iron organisms

Anti-Sph antibodies bound significantly to low-iron organisms of serovar Lai as visualized by confocal microscopy (Fig. 34). Low levels of fluorescence were detected in the high-iron organisms. This was evident in the immunofluorescence images (A1 and B1) and in the composite images (A3 and B3); with the counter stain propidium iodide showing the equivalent numbers of high- and low-organisms (A2 and B2). When anti-Sph antibodies were neutralized by prior incubation with rSph3, negligible binding of the antibodies to low-iron organisms (C1) was observed.

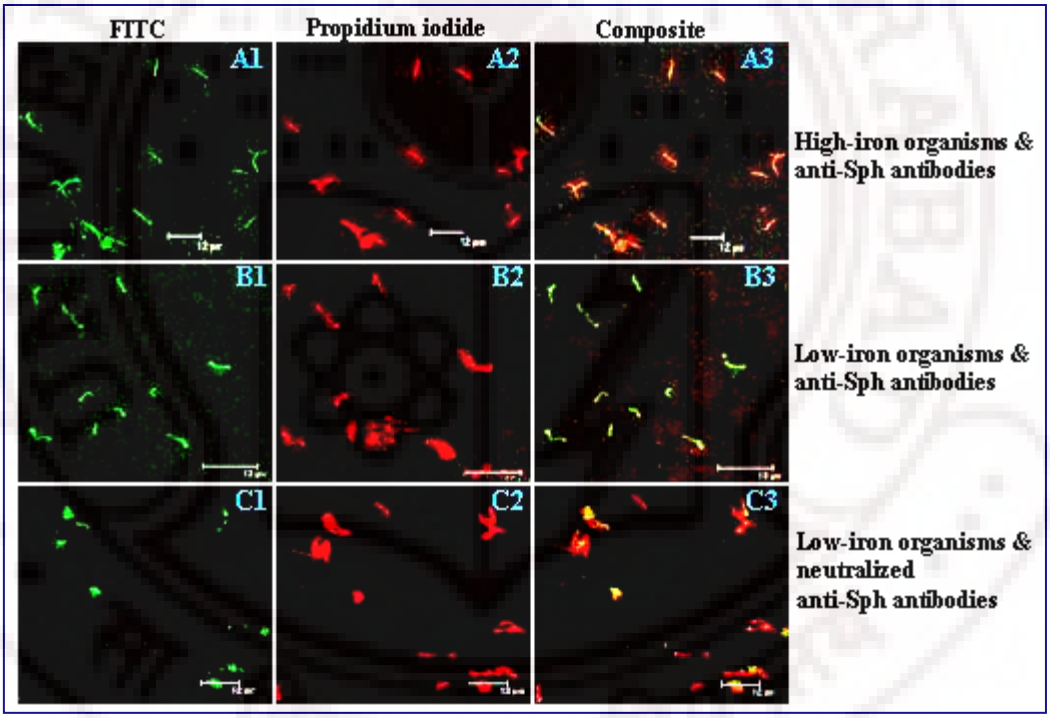


Fig. 34. Confocal microscopy: iron - regulated expression and surface localization of sphingomyelinase(s) in *L. interrogans* serovar Lai. Organisms cultured under high-iron (Panels A1 – A3) and low-iron conditions (Panels B1 – B3) were incubated with anti-Sph antibodies. An identical preparation of low-iron organisms was treated with neutralized anti-Sph antibodies (Panels C1 – C3). The immuno-reactivity was visualized using FITC - conjugated secondary antibody. Propidium iodide was used as DNA counter stain.

3.3.3.2. Detection of 63 kDa OMP with anti-Sph antibodies

When OMPs of serovar Lai grown under high- and low-iron conditions were resolved on 5 – 20 % gradient gel and subjected to immunoblotting, anti-Sph antibodies reacted significantly with two OMPs of approximate molecular masses of 63 and 60 kDa respectively (Fig. 35b, lanes 3 & 4). There was no difference in the reactivity between high- and low-iron cells. Incidentally, the serum from a patient with leptospirosis reacted strongly with these two OMPs (Fig. 35c; lanes 5 & 6). The serum sample was positive by MAT with the infecting serovar identified as serovar Lai.

The 63 kDa protein was purified by preparative gel electrophoresis, followed by subsequent gel elution procedure (Fig. 35d, lane 3) and was subjected to immunobloting with anti-Sph and neutralized anti-Sph antibodies. A significant reactivity was noticed with anti-Sph antibodies (Fig. 35e, lane 4) and no reactivity was seen with neutralized anti-Sph antibodies (Fig. 35f, lane 5). The OMPs of high- and low-iron organisms were subjected to EZ view red protein A agarose gel conjugated with anti-Sph antibodies. The 63 kDa protein band was immunoprecipitated under both high- and low-iron conditions using anti-Sph antibodies (Fig. 36).

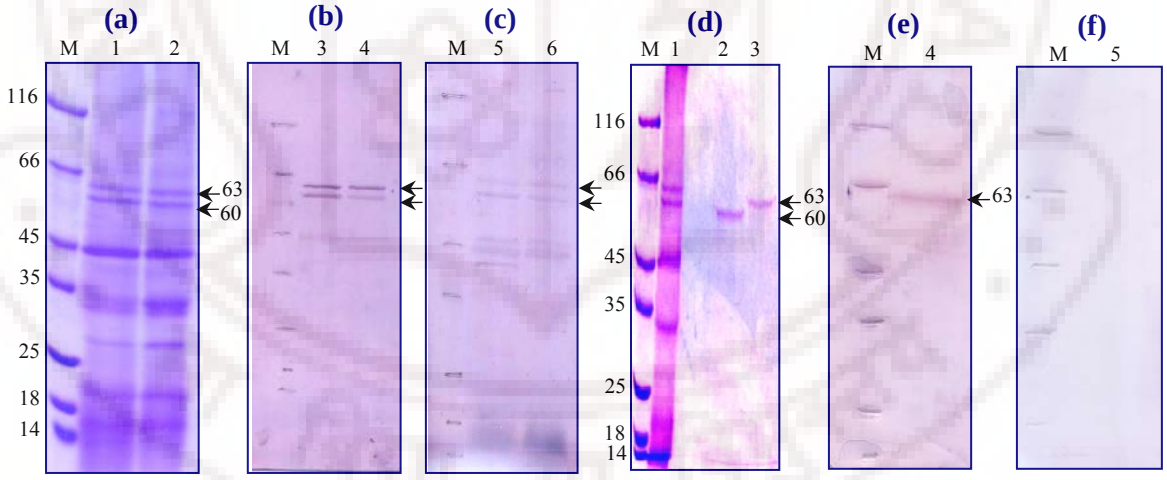


Fig. 35. Immunoreactivity of anti-Sph antibodies with two outer membrane proteins of *L. interrogans* serovar Lai.

OMPs of high- (lane 1) and low-iron (lane 2) organisms of serovar Lai were analysed by SDS-PAGE (Panel a) and by immunoblotting with anti-Sph antibodies (Panel b) and MAT – positive serum from a patient (Panel c).

Panels (d, e and f) represent the purified OMPs detected by anti-Sph antibodies. Panel (d) represents the SDS-PAGE of the purified 63 and 60 kDa proteins (lanes 2, 3). Panel (e) shows the immunoreactivity of the purified 63 kDa protein with anti-Sph antibodies (lane 4) and Panel (f) shows the failure of Sph-neutralised anti-Sph antibodies to react with an identical blot as in lane 5 (Panel e), thus confirming the specificity of the immunoreactivity.

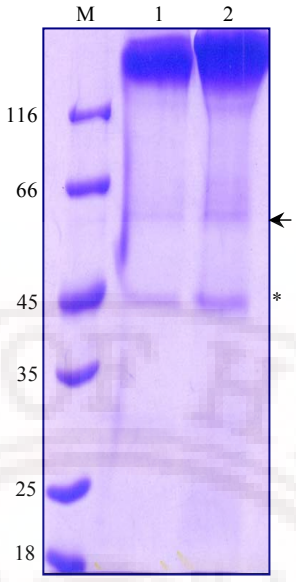


Fig. 36. SDS-PAGE analysis of the 63 kDa OMP immuno-precipitated with anti-Sph antibodies. OMPs of high- (lane 1) and low-iron (lane 2) organisms of serovar Lai were subjected to immuno-precipitation with anti-Sph antibodies bound to EZ-view red protein A - agarose beads. The washed beads were subjected to SDS-PAGE. Lane M is the protein molecular weight marker and * indicates the immunoglobulin heavy chain.

3.3.3.3. Characterization of 63 kDa OMP

a. Sequence analysis

The purified 63 kDa OMP was sequenced by tandem mass spectrometry. Three distinctive peptides were obtained (Fig. 37a), which unambiguously identified the protein as an outer membrane efflux protein (LA0957) in the genome of serovar Lai. Fig. 37b shows the TolC and outer membrane efflux protein domains in the 63 kDa protein, as analyzed by the NCBI domain search program.

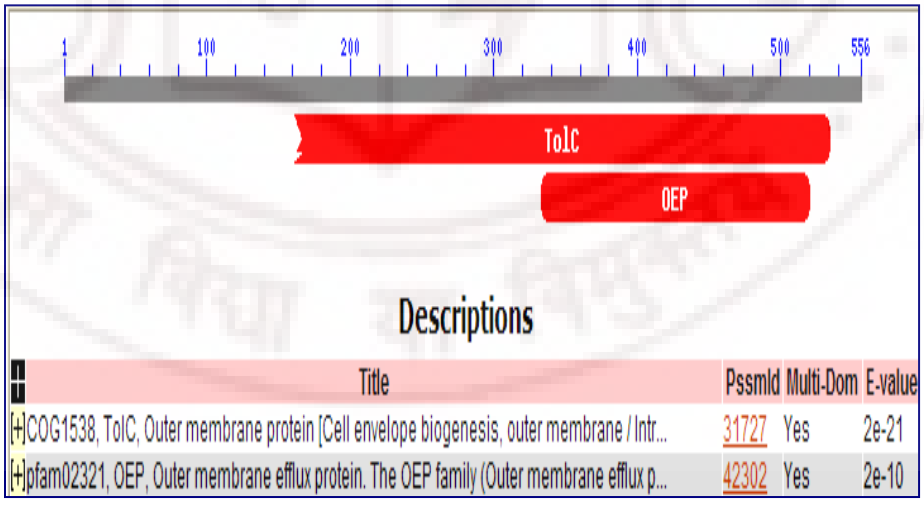
The genome of *L. interrogans* serovar Lai encodes several outer membrane efflux proteins, namely LA1100, LA1445, LA0581, LA3733 & LA3927; the 63-kDa protein (LA0957) is henceforth termed as $TolC^{63}$. $TolC^{63}$ showed high similarity with LA1100 of serovar Lai (Fig. 37c). When analyzed against other bacterial genomes, $TolC^{63}$ showed highest homology with the corresponding orthologs in L550 and JB197 strains of *L. borgpetersenii* serovar Hardjo (alignment score of 85.4809) and non-pathogenic *L. biflexa* serovar Patoc (ames strain) (alignment score of 46.6786). A low level of similarity was seen with the TolC proteins of the *Azoarcus* spp. (alignment score of 11.6438), *Neisseria meningitidis* (alignment score of 11.7773) and *Rhodobacter* spp

(alignment score of 12.0172). The phylogenetic tree (Fig. 38a) shows *L. interrogans* serovar Lai, *L. borgpetersenii* strains JB197, L550 and *L. biflexa* strain Patoc-I, *Campylobacter* spp., Flavobacteriales and *Bdellovibrio* spp. belong to a single cluster, with the TolC proteins of *E. coli, Neisseria meningitidis, Sphingomonas, Pseudomonas* and *Vibrio parahaemolyticus, Alcanovorax* spp. and *Rhodobacter* spp. forming a second major cluster (Fig. 38a). TolC[63] had corresponding orthologs in the genome of *L. borgpetersenii* serovar Hardjo (LBJ2313 & LBL0794) (Fig. 38b).

(a)

MYYKKKRTLEKFLVFGTILLTMIQPTWSEDILPEETVLEENKKSP
VENLGDSKKILRLTLKDAVNYVLEKNITIQNAKMEYVKADGGELK
NESQFTWNLIGGITVFRTTLPANRNNIFAGTKQSQDKLSVGIEKN
FRTGTYAKLEASTTRFDTSAFENPSTTPSNLAALAIPPLYTGALT
ITLSQEILKYSFGKTQKEREAILRQNTVIKREELIYVLSQLVAQT
LIQYWSLNIYDSNVK**TLQDLESNTR**NIRDLTVRKRNLGLSEGFEV
NLWNSILSQTAGNLEKAKVSRKEAERNLIRILNADPSSKIEGVTD
LQENVPLDFNVEKDYIYALDHRTDLKNLRKQREIAELNLKIKEAE
DMPSLKLSGAYSTR**GQNIVSPQQNLTDGNR**GVASFKYPEAYAAFQ
FSYPLWDKGIKADIRNAKLDVQNLEKKEAELKLSIKEELENRYAA
IVADKDIFEGAKKRKEEANKFYKGLSERFRQGRFTAVAVKNALDN
VIQSELQVTQAKIQLNIDILRYELAKNHIFER**FGVNVNDIIDR**LM
KMVDIAQSKSSTETSEK

(b)



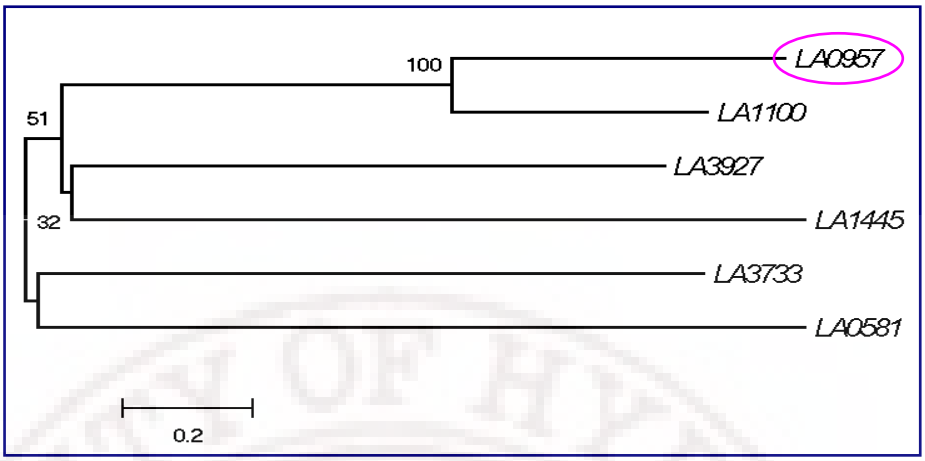
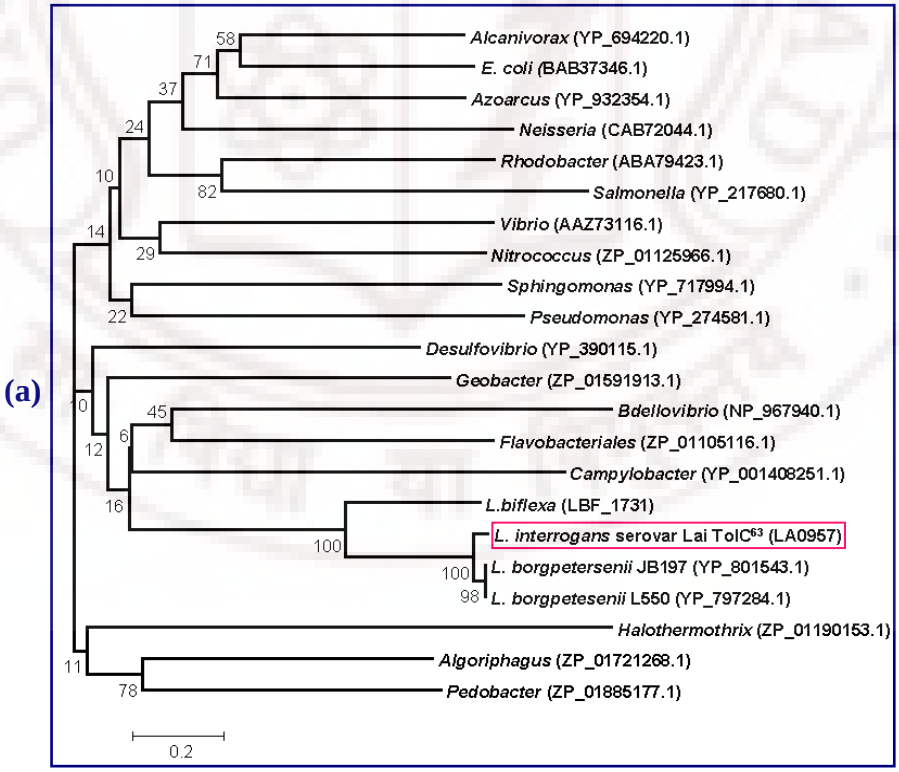


Fig. 37. Sequence analysis of the sphingomyelinase-associated 63 kDa outer membrane protein of *L. interrogans* serovar Lai.
Panel (a) shows the 63 kDa OMP identified by tandem mass spectrometry as the outer membrane efflux protein LA0957. The three tryptic peptides sequenced are represented in red (sequencing done by Prof. Friedrich Altmann, Austria is acknowledged).
Panel (b) is the NCBI domain search program showing the TolC and outer membrane efflux protein domains in the 63 kDa protein.
Panel (c) shows the five outer membrane efflux proteins of *L. interrogans* serovar Lai and highlights the relatedness of the 63 kDa protein (TolC^{63}) with LA1100 as compared to the other proteins.



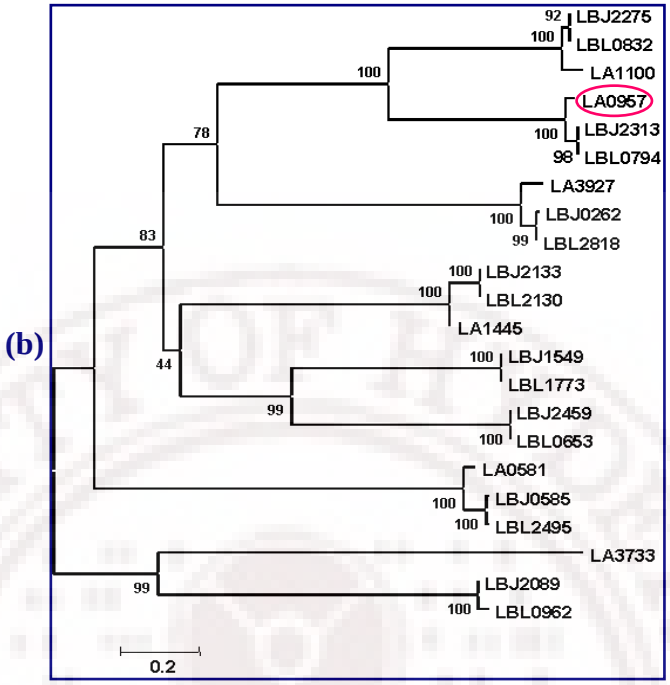


Fig. 38. Phylogenetic analysis of TolC^{63} of *L. interrogans* serovar Lai. Panel (a) is the dendrogram showing separate cluster of leptospiral homologues of TolC^{63} when compared to the corresponding orthologs in other bacterial genomes. Panel (b) is the dendrogram showing homology of TolC^{63} with the corresponding orthologs in *L. borgpetersenii* serovar Hardjo. The phylogenies generated by neighborhood joining with 400 bootstrap replicates, rooted at midpoint and bootstrap values, are shown as percentages. The numbers refer to the divergence between the sequences.

b. Homology modelling of TolC^{63}

The 3D structure of the modeled TolC^{63} protein (Fig. 39a) showed clear partitioning of a β-domain, an α-helical domain and a mixed α / β -domain as seen in TolC of *E. coli* (Fig. 39b). The monomer molecule consists of 4 strands representing the β barrel and 4 α helices. The structural alignment of TolC^{63} model with the crystal structure of the TolC protein of *E. coli* (Fig. 39c) showed high degree of structural similarity between these two proteins. The structure validation was determined by PROCHECK software, which showed that 86.7% of peptide φ–ψ angles fall within the favourable region of the Ramachandran plot**,** with 9.8% falling within the additional allowed region and 2.8% in the generously allowed regions. TolC^{63} comprises of two approximate structural repeats, namely repeat 1 consisting of amino acid residues 16 - 294 and repeat 2 consisting of amino acid residues 295 – 557 respectively (Fig. 40a & 40b). The unique I - region involved in hemolysin secretion was also predicted using

multiple alignment with TolC of *E. coli*. The region is highly similar in both serovar Lai and Hardjo, with negligible similarity in the non-pathogenic Patoc (Fig. 41).

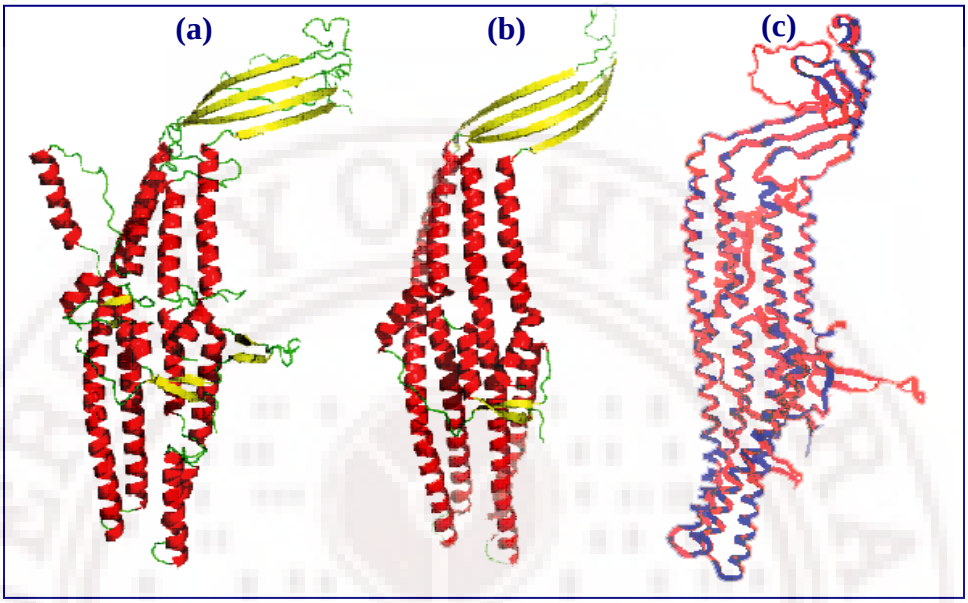


Fig. 39. Insight II homology modelling of TolC[63]. The homology module of Insight II software was used for modelling TolC[63] (a) using TolC of *E. coli* as the template protein (b). The structural superimposition (Panel c) of the crystal structure of (b) on the modelled structure of TolC[63] (a) shows high degree of similarity.

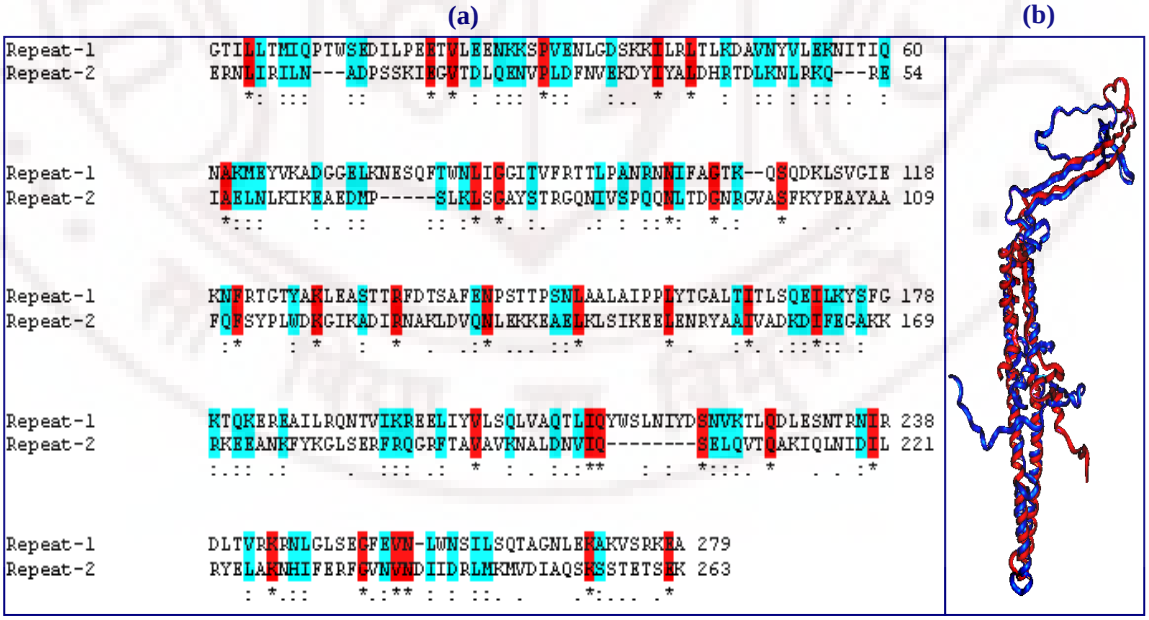


Fig. 40. Structural repeats in TolC[63]. Panel (a) shows alignment of amino-acid sequence of TolC[63] to highlight the structural repeats (shaded in blue and red). Panel (b) shows superimposed secondary structure of these structural repeats, with repeats 1 and 2 in blue and 2 respectively.

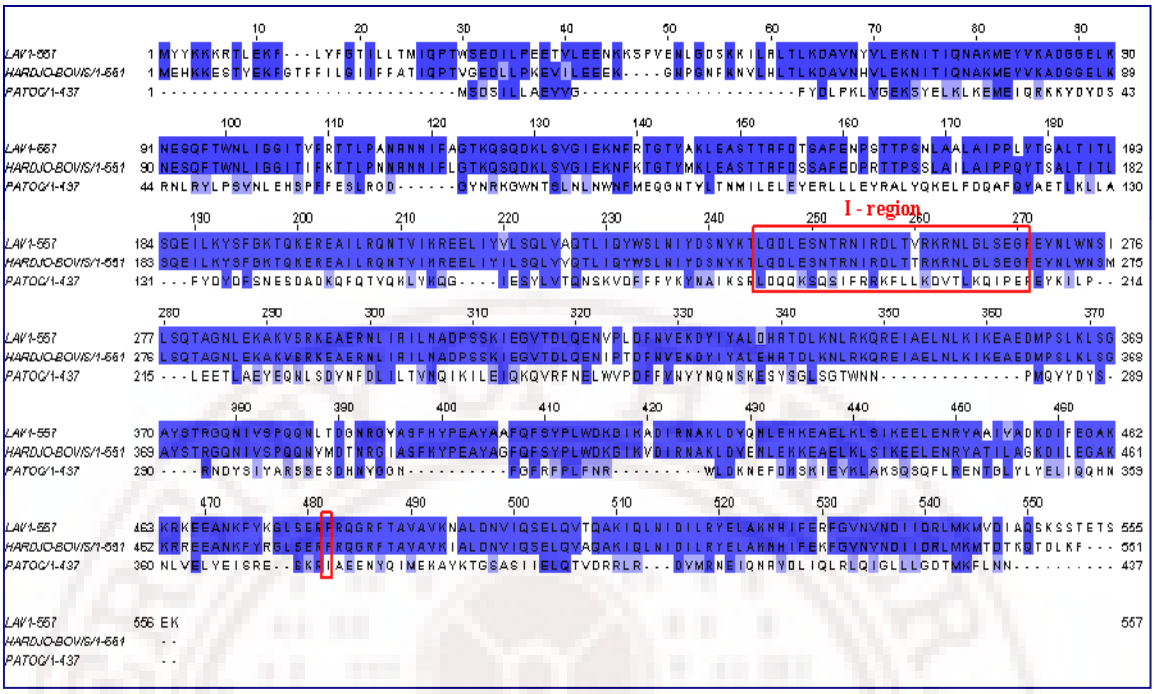


Fig. 41. *In silico* analysis and identification of the region associated with hemolysin secretion in TolC efflux proteins of pathogenic *Leptospira* spp. The sequence alignment shows higher homology of TolC[63] among the pathogenic leptospires as compared to the saprophytic *L. biflexa* serovar Patoc. The boxed region (amino acid residues 245 – 271) and the phenylalanine residue at position 482 represent the predicted region responsible for hemolysin secretion that is conserved in the pathogenic leptospires.

CHAPTER 4

DISCUSSION

4. Discussion

Bacterial pathogens have evolved diverse systems for acquiring iron as reviewed earlier. Direct acquisition of iron from host iron-containing molecules is well-studied in several bacterial systems and the ability to utilize heme compounds is particularly important in pathogenic bacteria, as heme is one of the most abundant forms of organic iron in animals (Otto *et al*., 1992; Wooldridge & Williams, 1993). There is increasing evidence to show that *Leptospira* spp. acquire iron directly as studies in our lab showed no evidence of siderophore expression. The major focus of research in our lab has been the understanding of iron acquisition in these organisms and we presented the first report on direct acquisition by a hemin-binding protein HbpA. As establishment of conditions of iron-deprivation proved difficult, an *in silico* approach showed a putative TonB-dependent outer membrane receptor protein (Sritharan *et al*., 2005) in *L. interrogans* serovar Lai. Spectrophotometric and spectrofluorimetric studies with recombinant HbpA confirmed this protein to be a hemin-binding protein.

In the present study, we established conditions of iron-limitation for the growth of *L. interrogans* serovar Lai and identified the 81 kDa HbpA in low-iron organisms, with no expression in the corresponding high-iron organisms. The iron-regulated and surface expression of this protein was further evident in immunofluorescence studies with anti-HbpA antibodies. We also identified a constitutively expressed 44 kDa hemin-binding protein that proved to be LipL41 by sequence analysis and immunoblotting studies with specific anti-LipL41 antibodies. Interestingly, HbpA is restricted to *L. interrogans*. The diagnostic potential of HbpA is evaluated in this study using immunological and molecular methods. As iron levels influence not only the iron acquisition machinery, but also virulence determinants, we analyzed the effect of iron on the expression of leptospiral sphingomyelinases and showed the iron-dependant expression and the extracellular release of a 42 kDa sphingomyelinase in outer membrane vesicles (OMVs) from low-iron organisms. We also identified an outer membrane efflux protein, TolC[63] associated with sphingomyelinase secretion in serovar Lai.

4.1. Iron-regulated expression of HbpA in *L. interrogans* species

Growth of pathogenic leptospires under conditions of iron-limitation was difficult, as they failed to grow in simplified low-iron media and additionally, were sensitive to iron chelators such as 2', 2' – dipyridyl, desferrioxamine and EDDHPA. Leptospires were tolerant to EDDA to a maximum concentration of 200 μM. The addition of EDDA was carefully standardized with the step-wise addition of the chelator to maintain optimal activity and viability of the organisms. The growth was regularly monitored and the organisms were analyzed for the 81 kDa protein. However, we were successful only upon a final incubation of the organisms at 37°C, thus implicating not only the iron-dependant but also the temperature-dependant expression of HbpA. It is highly likely that the protein is expressed under *in vivo* conditions within the mammalian host, where it is subjected to both iron-limitation and elevation of temperature as compared to the *in vitro* growth. It is to be noted that HbpA was seen in whole cell sonicates of the organisms with low levels of detection in outer membrane proteins (OMPs) preparations, which can be explained by their presence in the outer membrane vesicles (OMVs), as explained later.

Confocal microscopy revealed the significant binding of anti-HbpA antibodies to low-iron organisms that not only confirmed the iron-regulated expression of HbpA but also the surface association of the protein. The presence of the N-terminal signal peptide with the cleavage site located between amino acids 39 – 40 (IQA – QD) supports its surface expression. The corresponding high-iron organisms showed negligible fluorescence while the control smears of high- and low-iron organisms developed with antibodies against the constitutively expressed LipL41 showed equivalent fluorescence in both preparations. Our initial efforts in demonstrating HbpA expression by immunofluorescence were not successful when we incubated a suspension of live organisms with serum containing anti-HbpA antibodies. Subsequently, we used ammonium sulfate-precipitated anti-HbpA immunoglobulins instead of whole serum. This was due to agglutination and disintegration of the organisms in the presence of whole serum, which we demonstrated in a MAT assay (not shown here). Our observations have been strengthened by other reports. Using software programs such as PSORTb, ProtCompB and Proteome analyst (PA), Viratyosin *et al*. (2008) showed that

HbpA was unambiguously expressed on the leptospiral cell surface, while Yang *et al*. (2006), using both *in silico* (P-CLASSIFIER) and microarray analyses of *L. interrogans* showed that HbpA is outer membrane protein, among the 226 potential vaccine candidates. In the study published by Lo *et al*. (2006) on temperature-regulated leptospiral proteins, HbpA was not identified as it regulated not only by temperature but also by iron levels. Murray *et al.* (2008), using real time PCR showed that HbpA was an iron-regulated protein.

In addition to the HbpA, a 44 kDa hemin-binding protein is expressed by *L. interrogans* serovar Lai. It is not clear as to why another hemin-binding protein is expressed by this organism. It was interesting to observe that the protein is LipL41, a major outer membrane protein in several pathogenic serovars. Unlike other reports, we detected the protein even in the serovar Patoc. The finding that this protein is constitutively expressed and that HbpA is regulated by iron strongly suggests that the latter confers an advantage for the organism under *in vivo* conditions, and possibly is associated with virulence of the organism, as indicated by the role of iron levels on sphingomyelinases. *Treponema denticola*, a closely related spirochete showed a constitutively expressed 47 kDa hemin-binding protein (Scott *et al*., 1993) and two iron-regulated hemin-binding proteins, a 44 kDa HbpA and 42.8 kDa HbpB (Xu *et al*., 2001). It is well known that the mammalian host limits the amount of free iron by a process known as nutritional immunity (Kochan, 1976). It is therefore likely that these pathogens have adapted successfully to the low-iron conditions *in vivo*.

HbpA is a novel protein that is restricted to serovars belonging to *L. interrogans* species. Earlier studies (Sritharan *et al*., 2005) by PCR with specific HbpA primers and Southern analysis showed that the gene could be detected only in *L. interrogans*. In this study, other species were checked for the expression of HbpA by growing them under conditions of iron-limitation. HbpA was not expressed in *L. borgpetersenii* serovar Tarassovi strain Perepelitsin, *L. kirschneri* serovar Grippotyphosa strain Moskva V, *L. santarosai* serovar Sarmin strain CZ390 and non-pathogenic *L. biflexa* serovar Andamana strain CH11 as analyzed by immunoblotting with anti-HbpA antibodies. Comparative genomics of *L. interrogans* and *L. borgpetersenii* clearly implied the better survival of the former, with its larger genome (Nascimento *et al*., 2004; Bulach *et al*.,

2006). Whether HbpA contributes to the survival and virulence of serovars of *L. interrogans* remains to be understood. It also remains to be understood as to how the other serovars acquire iron. In *E. coli*, about six iron uptake systems are reported (Neilands, 1990; Griffiths & Chart, 1999). The preliminary observations in this study on the 42 kDa transferrin-binding protein in *L. kirschneri* serovar Grippotyphosa strain Moskva V warrants further investigations in other leptospiral serovars.

HbpA probably transports hemin by a mechanism similar to that of other Fe^{3+} - siderophore / hemin receptors. HbpA shows similar β-barrel structure with three domains namely the β-barrel, plug domain and N-terminal TonB box. The β-barrel is kept closed by the plug; upon binding of the ligand to specific residues on the loops extending from the β-barrel, conformational changes are thought to occur that plug domain to move thus opening the outlet from the barrel and allowing the entry of the iron, either as free iron or as iron bound to hemin. Further studies are required to show the internalization of the iron from the hemin. The energy for this internalization is highly likely to be similar to that of other ferric-siderophores as HbpA, like other ferric-siderophore receptors is a TonB-dependant protein acting in concert with ExbB and ExbD, as the genes encoding these two proteins are present in the genome of serovar Lai.

There are reports by other researchers on the influence of iron on leptospiral proteins. Cullen *et al.* (2002), in their study on the global analysis of OMPs under different environmental conditions reported that LipL36 and pL50 could not be detected in leptospires grown under conditions of iron-limitation and at temperatures above 30°C. Their observations on LipL32 merit consideration, as they identified a number of cleavage products of LipL32 by 2D-gel electrophoresis; cleavage of LipL32 was observed only in high-iron organisms and not in low-iron organisms. Another group studied iron acquisition in *L. biflexa* (Louvel *et al*., 2005 & 2006). Their initial study identified 5 hemin requiring mutants in *L. biflexa* using random insertional mutagenesis. Three of these mutants had insertions in a gene encoding a protein that shares homology with the TonB-dependent ferric citrate receptor FecA of *E. coli* while the other two mutants showed a *Himar1* insertion into a *feoB* like gene. FeoB has been implicated to plays a role in uptake of ferrous iron in several bacteria. They also showed that

Leptospira could utilize exogenous siderophores; aerobactin and ferrichrome were used by both *L. biflexa* and *L. interrogans* while desferrioxamine was only used by *L. biflexa*.

Murray *et al*. (2008) in their efforts to demonstrate a functional heme oxygenase in *L. interrogans* serovar Manilae studied the effect of different iron sources on the expression of LA0706, LA1356, LA2641, LA3468 (TonB-dependent receptors), LA1857, LA2887, LB183 (Fur family) and LB191 (HbpA). Their study revealed that the expression of LA0706 and LA1356 was unaffected by varied iron conditions. LA1857, LA2887, LB183 and LA2641 were weakly suppressed in the presence of haemoglobin. Expression of HbpA and LA3468 was stimulated in the presence of haemoglobin, but only in the absence of ferrous iron, while under these conditions *hemO* was strongly up-regulated and LB011 (*hemCD*) appear to be co-regulated. This study strongly supports our experimental outcome that low-iron environment acts as a signal for the expression of HbpA.

Our earlier observations with the cloning and expression of the full-length *hbpA* showed that these clones showed poor growth as visualized on agar plates. As our objective was to express rHbpA in sufficient quantities for screening purposes, a 1449 bp region of *hbpA* was cloned and expressed as a 55 kDa recombinant protein. The $rHbpA_{55}$ protein retained the FR (A/P) P – NPNL motif that was responsible for hemin-binding as shown by the binding of this protein to hemin-agarose beads. Antibodies were raised in rabbits and further studies were done to evaluate the diagnostic potential of this antigen in the screening of clinical samples.

4.2. Diagnostic potential of HbpA

The diagnostic potential of HbpA was evaluated by molecular and immunological methods using *hbpA*-based PCR and $rHbpA_{55}$-based sandwich ELISA assay. PCR done with DNA from reference strains of *Leptospira* spp. confirmed that *hbpA* was present only in serovars belonging to *L. interrogans*, as reported by us earlier (Sritharan *et al*., 2005). This was then extended to clinical isolates obtained from different geographical locations. Among the 91 clinical isolates, 51 were positive for *hbpA* and interestingly all these samples were from serovars belonging to *L. interrogans*. None of the other serovars belonging to other species, namely *L. santarosai*, *L. meyeri*, *L. kirschneri*, *L. noguchii*

and *L. borgpetersenii* showed any 1449 bp product with the specific primers. The most satisfying part of our observations in this study has been the fact that all *L. interrogans* clinical isolates, previously typed by MLST (Ahmed *et al*., 2006) were also unambiguously identified as *L. interrogans* by the *hbpA*-based PCR. This 100% concordance of a single step PCR to complicated typing schemes such as MLST would greatly simplify its use in reference laboratories and in the field for rapid identification and outbreak characterization. Another important observation made in this study is the high reproducibility of our PCR method in detecting *L. interrogans* DNA originating from strains circulating in diverse geographical regions and from different host species.

Thus, the *hbpA*-based PCR could be a useful tool for the detection of serovars belonging to *L. interrogans*. Till date, several PCR-based methods have been reported with merits and demerits. PCR based on 16S rDNA sequence (Hookey, 1992; Shukla *et al*., 2003), G1/ G2 primer-specific method (Gravekamp *et al*., 1993) and real-time PCR (Smythe *et al*., 2002) have been used on clinical samples for diagnosis of leptospirosis. A recent publication includes the *ompL1*-based PCR (Reitstetter, 2006), detects *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. santarosai*, *L. weilli* and *L. noguchii* without cross-reacting with other leptospiral serovars. It would be interesting to develop multiplex PCR using different combinations of primers for the effective diagnosis of leptospirosis.

55 serum samples from patients with leptospirosis were screened by MAT, LeptoTek Dri-Dot and IgM ELISA in a retrospective study of cases from a hospital in Hyderabad. The LeptoTek Dri-Dot identified 52 cases as positive and can be attributed to the higher level of anti-leptospiral antibodies in these patients who came for treatment late in the disease. This probably would not be the case if LeptoTek Dri-Dot was used in a prospective study for screening a population at risk, as the titres of antibodies would be low and may not be found positive in this test (as mentioned in the kit). 28 of the 32 samples tested by IgM ELISA were positive and 50 of the 55 serum samples showed anti-leptospiral antibodies by MAT analysis. A comparative analysis of LeptoTek Dri-Dot with MAT showed relatively high sensitivity (96%) of this test with a positive predictive value of 87%. The sensitivity of IgM ELISA as compared to MAT was found to be lower (86.7%).

Leptospira interrogans serogroup Icterohaemorrhagiae was identified as the predominant serogroup (62%) by MAT. There was a similar report from Mumbai and Pune (Bharadwaj *et al*., 2002), while *L. interrogans* serovar Grippotyphosa was demonstrated in Andamans and Kerala (John, 1996). In a study in Tamil Nadu, the serogroup Autumnalis was found in Chennai, Cumbum and Tirunelveli, Panama in Madurai and Icterohaemorrhagiae in Bodi (Ratnam, 1994). We also screened bovine samples from villages of Mahaboobnagar district of Andhra Pradesh. Among 30 samples analyzed by MAT, *L. interrogans* serovar Pomona was prevalent in this region followed by Ballum, Australis and Lai. It is well known that leptospirosis is widespread in farm and domestic animals in many parts of India including the north-east, West Bengal, Bihar, Madhya Pradesh, Maharashtra, Andhra Pradesh, Karnataka, Kerala, Tamil Nadu, Punjab and Haryana (Ratnam, 1994). As re-iterated by John (1996), fewer reports on leptospirosis do not imply that this disease is prevalent only from these reported areas. There is a need for etiologically diagnosing leptospirosis in all hospitals as this would increase public awareness and help to deal with situations of outbreaks, as was recently witnessed in the Mumbai floods and would definitely decrease the mortality associated with this disease. This would be possible only with the development of a diagnostic kit that is made available in all peripheral hospitals.

Leptospirosis at present is grossly under-reported due to lack of simple, rapid and efficient tests for early diagnosis. Isolation and demonstration of leptospires, serological techniques for detection of antibody and PCR are some of the available laboratory methods for diagnosis discussed earlier. Low success rate of isolation, unreliability of direct demonstration of leptospires using dark field microscopy and inaccessibility of molecular techniques like PCR-based diagnosis to most of peripheral hospitals and clinics make serological tests play an important role in the diagnosis of leptospires (Faine, 1982). Though the Microscopic agglutination test (MAT) continues to remain the "gold standard" for diagnosis, it has its limitations. The immense serovar diversity among the pathogenic leptospires necessitates the maintenance of a large battery of serovars, thus restricting the use of the test. The test is also complex to perform and interpret. The lack of good and user friendly diagnostic test has led to a plethora of tests being developed which includes Latex Agglutination (LA), Haemagglutination Assays (HA),

LeptoTek Dri-Dot, Lepto dipstick, Lepto lateral flow, Microcapsule agglutination test (MCAT), IgM ELISA and dot ELISA. All these commercial kits are to be imported and costly. Moreover, they were found to be more sensitive during acute stage of the disease after the onset of symptoms. So, there is a need for developing a cost effective, easy-to-do and indigenous diagnostic test that would be more sensitive during the early stages of disease.

Dot blot analysis was done preliminarily using HbpA as antigen and the highest reactivity was seen with serum that was positive by MAT for the serovar Lai. HbpA was subsequently used in an ELISA format for the detection of anti-HbpA antibodies. Anti-HbpA antibodies were initially purified by subjecting to immobilized *E. coli* lysate column for selective removal of *E. coli* IgG contaminants. The purified antibodies, tested for cross-reactivity with whole cell sonicates of commonly infecting human bacterial pathogens showed that negligible immunoreactivity with other bacterial antigens. Sandwich ELISA was standardized by determining the optimal antigen concentration and antibody dilution by checkerboard titrations. Using a cut-off value of 0.4497, calculated from healthy controls, the test performed on 50 human serum samples showed positivity in 80% of the MAT positive serum samples. The sensitivity and specificity of the test were 87.18% and 81.81% respectively. The reduced sensitivity and specificity of the test, when compared to MAT was probably due to assay of only the IgG response and could also be due to antibodies against other leptospiral serovars because of mixed infection. However, its sensitivity and specificity were found to be higher than that reported by Flannery *et al.* (2001), who used recombinant LipL32, OmpL1, LipL41 and Hsp58 antigens in IgM- and IgG-based ELISA. They found that the sensitivities were 56%, 16%, 24% and 18%, respectively during acute phase and 94%, 72%, 44% and 32% during convalescent phase. The diagnostic potential of several OMPs have also been analysed by other research groups that included LipL41 (Flannery *et al*., 2001; Mariya *et al*., 2006; Senthilkumar *et al*., 2007), LipL32 (Flannery *et al*., 2001; Fernandes *et al*., 2007) and Lig proteins (Croda *et al*., 2007; Srimanote *et al*., 2008). Senthilkumar *et al.* (2007) conducted rLipL41 based IgG ELISA and reported that the sensitivity and specificity of the test were 83.33% and 93.07%. Croda *et al*. (2007) conducted LigB-based ELISA and found that the specificities of the test were 93 - 100% and 90 - 97%

among sera from healthy individuals and patients with symptoms suspected of leptospirosis. Srimanote *et al*. (2008) performed LigA-based IgM and IgG ELISA on acute and convalescent sera of patients with leptospirosis and found that the specificities were 98% and 100% respectively.

Our findings with bovine sera were not very satisfactory. The cut-off value for this assay was calculated as 0.2656. Among 50 bovine sera screened, 40 (80%) were positive. The specificity, sensitivity, accuracy and positive predictive value of the ELISA in relation to standard MAT were 78.95%, 16.67%, 64% and 75% respectively. This test was less sensitive and specific for bovine sera when compared to human sera. The probable reason might be due to the fact that the bovine population acts as reservoirs for leptospires with infection by different serovars.

4.3. Iron levels and virulence determinants in *Leptospira* spp.

Genome analysis revealed the presence of several virulence determinants in *Leptospira* spp. (Ren *et al*., 2003; Nascimento *et al*., 2004). Although virulence factors such as hemolysins (Kasarov, 1970; Thomson & Manktelow, 1986), lipopolysaccharide (Isogai *et al*., 1986), glycolipoprotein (Alves *et al*., 1992), peptidoglycan (Dobrina *et al*., 1995), heat shock proteins (Stamm *et al*., 1991), flagellin (Goldstein & Charon, 1990) and others may contribute to the pathogenesis, their mechanisms of pathogenesis are not understood. With increasing information from genome analysis of several *Leptospira*, genome profiling and comparative genomics will help in the deciphering the role of these molecules.

Reports on leptospiral hemolysins are varied with the first report by Alexander *et al.* (1956). Leptospiral hemolysins were shown to exhibits phospholipase A and sphingomyelinase A activities, the latter being restricted to pathogenic serovars (Kasarov, 1970; Bernheimer & Bey, 1986). Segers *et al*. (1990) cloned and characterized a sphingomyelinase gene (*sphA*) from serovar Hardjo and later, based on hybridization studies showed that sphingomyelinases were present in all pathogenic members and were notably absent in the saprophytic members of *Leptospira* spp. (Segers *et al*., 1992). Lee *et al*. (2002) characterized SphH as a pore-forming hemolysin that lacked both sphingomyelinase and phospholipase activities. Recently, Hauk *et al*. (2005) studied the

hemolytic activity of HlyX hemolysin and the potentiating effect of LipL32 on hemolysis.

Iron is known to be a regulatory molecule for the expression of toxins and virulence determinants in a number of bacterial systems. The diphtheria toxin, shiga toxin, aerobactin and others are expressed only conditions of iron limitation, highlighting the contributory role of iron as one of the virulence determinants (Griffiths & Chart, 1999; Sritharan, 2000 & 2006). In this study, the role of iron on the expression of hemolysins in the serovar Lai was analyzed. There are as many as 10 hemolysin genes in the genome of serovar Lai. Sequence alignment and phylogenetic analysis of these hemolysin genes was done. All the non-sphingomyelinases (LA3937, LA0378, LA0327, LA1650 & LA0177) formed a single cluster and sphingomyelinases (LA1027, LA1029, LA4004, LA3540 & LA3050) formed a separate cluster. When compared to the sphingomyelinases of serovar Hardjo, it was found that strain JB197 contained 3 corresponding orthologs and strain L550 had 2 orthologs. The non-pathogenic *L. biflexa* serovar Patoc had no corresponding orthologs to the sphingomyelinases but carried the genes encoding five putative hemolysins with phospholipase activity.

Sphingomyelinases are probably being involved in the typical vascular damage seen in acute leptospirosis in humans with symptoms of either pulmonary haemorrhage and / or involvement of liver with associated jaundice. These sphingomyelinases could release heme and haemoglobin from host red blood cells, thereby ensuring a readily available source of iron for the pathogen. Considerable amount of the released hemin can be taken up by the pathogenic *Leptospira* via the hemin receptors, even though the host proteins rapidly bind hemin and help to clear these molecules from the circulation. Much remains to be understood about the regulation of expression and mechanism of secretion of these toxins into the extracellular milieu. In *E. coli* and several Gram-negative bacteria, the hemolysins are transported by the Type I secretary pathway, with the TolC outer membrane efflux protein playing an important role in the transport process. Surprisingly, it has also been described that a fraction of the toxin is associated with the bacterial cell surface (Oropeza-Wekerle *et al*., 1989) with a considerable proportion of the toxin being released as outer membrane vesicles (OMVs) to the outside in a physiologically active form (Balsalobre *et al*., 2006).

In several Gram-negative bacteria, the transport of secreted proteins to the extracellular milieu involves the formation of outer membrane vesicles (OMVs), which are complexes formed by association of the proteins with lipid membrane structures (Wai *et al*., 2003) (Fig. 42). There is a constant formation and release of these OMVs. Several protein toxins are released via these OMVs to the outside, as exemplified by the α - hemolysin / HlyA of *E. coli*

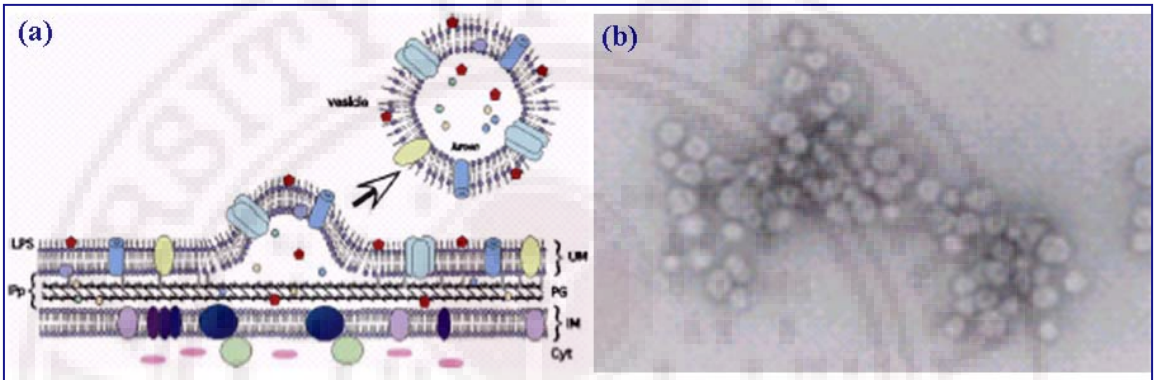


Fig. 42. Model of vesicle biogenesis. OMVs are proteoliposomes consisting of OM phospholipids and LPS, a subset of OMPs and periplasmic proteins. Vesicles are likely to bud at sites where the links between peptidoglycan and OM are infrequent, absent or broken (panel a). Panel (b) show the OMVs produced by *E. coli*. LPS – lipopolysaccharides, Pp – Priplasm, OM – outer membrane, PG – Peptidoglycan, IM – inner membrane and Cyt – cytosol.

Our objective was to screen the spent growth medium for the presence of sphingomyelinases using specific anti-sphingomyelinase antibodies that we raised against rSph3. However, the results were not conclusive following which we obtained the OMVs of high- and low-iron organisms by ultracentrifugation and subjected them to protein analysis and immunoblotting with anti-Sph, anti-HbpA and anti-lipL32 antibodies. Our assumption of finding sphingomyelinase in OMVs was based on two observations: first, several bacterial protein toxins are released via OMVs, for *eg*. α - hemolysin of *E. coli* and second, leptospires produce OMVs (Haake & Matsunaga, 2002; Nally *et al*., 2005). We obtained a five-fold bigger pellet of OMVs from the spent growth medium of low-iron cultures of serovar Lai. The release of the OMVs from the bacterium and the ultrastructure of individual vesicles was analyzed by transmission electron microscopy (TEM) and visualized by 1% uranyl acetate stain. Anti-sphingomyleinase antibodies

reacted strongly with a 42 kDa protein. The smaller size of the sphingomyelinase (LA4004 encodes a protein of predicted size of 65.33 kDa) implies C-terminal processing of the protein, also reported by others (Segers *et al*., 1990; Zhang *et al*., 2005). Interestingly, anti-HbpA antibodies detected the iron-regulated 81 kDa hemin-binding protein HbpA that strongly supported our hypothesis that iron-limitation induced the expression of sphingomyelinase, as HbpA expression is an indicator of iron-limitation. It is interesting that a 29 kDa band also reacted strongly with anti-HbpA antibodies and ongoing work focuses on the characterization of this protein. Detection of LipL32 served as a control, as this 32 kDa outer membrane protein, specific for pathogenic leptospires was seen in both high- and low-iron cultures. The significance of the relatively lower level of the protein seen under low-iron conditions remains to be understood. LipL32 has been the subject of research in several laboratories. It was demonstrated to be a major antigen *in vivo* during leptospiral infection (Haake *et al*., 2000), implicated in interstitial nephritis in proximal tubular cells (Yang *et al*., 2002) and of greater importance, shown to promote hemolysis of RBCs and hence referred to as hemolysis-associated protein (Hap-1). The exact role of LipL32 in hemolysis is however not clear. Of significance is the finding that none of the above three proteins, namely the sphingomyelinase, HbpA and LipL32 are expressed by the non-pathogenic *L. biflexa* serovar Andamana, thus implying the role of these proteins in the survival of the pathogenic serovar Lai.

To further investigate if the sphingomyelinase hemolysin associated with OMVs of serovar Lai was active, a quantitative hemolytic assay was performed. About 17% hemolysis was observed with OMVs from high-iron organisms, though it was 2.3 fold lower than that seen with the OMVs of low-iron organisms. Anti-Sph antibodies caused a 3.2 to 3.4-fold reduction of the hemolytic activity in OMVs of both high- and low-iron organisms and did not abolish the activity totally. This clearly indicates that other hemolysins are expressed while the iron-regulated sphingomyelinase seen in this study is also expressed at low levels in iron-replete organisms. Furthermore, hemolytic activity was increased in the presence of calcium (10mM), which is consistent with the fact that the activity of the hemolysin is calcium-dependent. In this context, it may be mentioned that there are reports (Picardeau *et al*., 2008; Segers *et al*., 1990) that non-pathogenic *L.*

biflexa also showed hemolytic activity, that can be associated specifically with the hemolysins, as this serovar does not elaborate the sphingomyelinases.

Reports on the secreted α-hemolysin (HlyA) from *E. coli* showed that a fraction of the secreted HlyA remained on the bacterial cell surface (Balsalobre *et al*., 2006; Oropeza-Wekerle *et al*., 1989). In this study, we demonstrate the association of sphingomyelinase on the cell surface of serovar Lai by immunofluorescene studies with anti-Sph antibodies. Immunofluorescence studies using confocal microscopy showed that low-iron organisms of the serovar Lai reacted strongly with anti-Sph antibodies, demonstrating not only the iron-regulated expression of these toxin molecules but also their association with the cell surface. The latter is not an unexpected finding, as several secreted proteins remain associated on the cell surface before release into the immediate environment. The specificity of binding of the anti-Sph antibodies to sphingomyelinase was evident from the significant reduction of the antibody-associated fluorescence when the antibodies were neutralized by pre-absorption with rSph3. The low level of fluorescence seen in high-iron organisms can be due to two possibilites. First, it is likely that sphingomyelinase is expressed at low levels even under high-iron conditions established in this study and second, one or more of the other hemolysin(s) are probably expressed constitutively, unaffected by iron levels.

4.4. TolC protein and the possible role in the transport of sphingomyelinase

Immunoblotting of outer membrane proteins and immuno-precipitation with anti-Sph antibodies bound to EZ-view red protein A agarose beads of both high- and low-iron organisms showed two immuno-reactive proteins of approximate molecular mass of 63 and 60 kDa respectively expressed constitutively. The 63 kDa protein was identified as an outer membrane efflux protein encoded by LA0957 in the genome of serovar Lai. The purified protein also reacted with anti-Sph antibody, while neutralized antibody did not react, indicating that the sphingomyelinase (and possibly the other hemolysins), transported to the outside by this protein was still associated with it. It was reported in *E. coli* that while most of the HlyA toxin molecules are secreted out, a fraction of the toxin molecules is still associated on the cell surface (Balsalobre *et al*., 2006; Oropeza-Wekerle *et al*., 1989).

The 63 kDa outer membrane efflux protein is referred to as TolC^{63} as it is one of the six outer membrane efflux proteins in serovar Lai. Bioinformatic analyses showed the high level of homology of TolC^{63} with the corresponding orthologues in *L. interrogans* serovar Copenhageni and *L. borgpetersenii* serovar Hardjo. Homology modeling done using Insight II Modeler showed that structurally TolC^{63} is similar to the TolC of *E. coli*. The polypeptide folds into a structure comprising of distinct β-barrel, α-helical and mixed α / β-domains. The crystal structure of the TolC of *E. coli* (Koronakis *et al*., 2000) consists of 3 such monomers (also called as protomers) that assemble as a homotrimer; the β-barrel of the three monomers form adjacent sheets and form 12 anti-parallel strands, with the respective domains of each monomer twisted to the right (Sharff *et al*., 2001). The 12 strands of the four α-helices, forming the main body of the protein are also oriented in an anti-parallel manner. However, unlike the β barrel, these α-helices are twisted to the left. The transition from the right-twisted β-barrel to the left twisted α-helical barrel is accommodated through abrupt turns in proline-containing inter-domain linkers, a feature conserved in the family of these bacterial outer membrane proteins. The β-barrel is located in the outer membrane and the α-helical barrel extends into the periplasm. The loops at the top of the β-barrel may act as a partial external lid and probably have conformational mobility. Inter-helical contacts occur in different parts of the α-helical barrel and the contact made by Tyrosine at 362 position is well conserved in the TolC family (Koronakis *et al*., 2000). A notable feature of the leptospiral TolC^{63} is the presence of phenylalanine residue at 482 position that aligned with Tyrosine 362, when compared by multiple sequence alignment (ClustalW) with *E. coli* TolC. Like other bacterial outer membrane efflux proteins (Koronakis *et al*., 2004; Sharff *et al*., 2001) TolC^{63} consists of two structural repeats. As the structural repeat corresponds to a repeat in the primary sequence of certain bacterial outer membrane efflux proteins (Johnson & Church, 1999), TolC is thought to have evolved by gene duplication from a common ancestor, which may have functioned as a hexamer (Koronakis *et al*., 2004). The monomers of TolC^{63} probably assemble in a similar fashion as in *E. coli* to form a functional trimeric molecule.

Type I secretion system has been well understood in the transport of α-hemolysin (HlyA) of *E. coli*. The *hlyCABD* genes located in an operon mediate synthesis, activation,

and secretion of α-hemolysin. *hlyA* encodes for a 110 kDa inactive pro-toxin, which is post-translationally modified into an active form by HlyC-mediated acylation at two lysine residues and transported out via Type I secretion system.

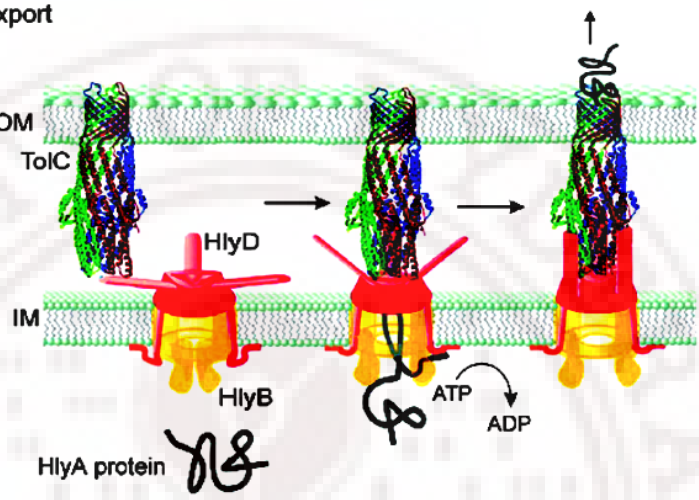


Fig. 43. Assembly of Type I secretion system and TolC-dependent export of hemolysin. A model indicating reversible interaction of outer membrane TolC with substrate-specific inner membrane complexes (translocases) containing an adaptor protein, HlyD and an energy-providing protein, HlyB. The transport occurs across the inner membrane (IM), outer membrane (OM), and the intervening periplasmic space (reproduced from Koronakis *et al*., 2004).

Type I system, utilizing a single, energy-coupled step for the transport of these molecules consists of three components, namely TolC, HlyB and HlyD (Fig. 43). It is thought that TolC and HlyD form a continuous tunnel capable of accommodating an unfolded protein during passage across both membranes of *E. coli*. Importantly, foreign proteins with the hemolysin (HlyA) signal sequence are also recognized and secreted by the HlyB-HlyD-TolC translocator and secretion efficiency significantly increases with longer C-terminal fragments, up to at least 218 amino acids (Kenny *et al*., 1991). Genetic studies have shown that removal of any one of the three components results in the abrogation of hemolysin secretion. The HlyA interaction with HlyB and HlyD may be simultaneous or sequential and probably represent the first steps of recognition, assembly of the machinery and the insertion of HlyA into the channel. After this HlyA–HlyB–

HlyD complex has been assembled at the inner membrane, a continuous channel is formed across the periplasmic space (Balakrishnan *et al.,* 2001). The toxin is released outside the bacterial cell without the formation of periplasmic intermediates (Blasalobre *et al*., 2006). The transport of α-hemolysin / HlyA of *E. coli* to the extracellular milieu involve the formation of OMVs (Wai *et al*., 2003)

We hypothesise that a similar mechanism of transport of the leptospiral sphingomyelinase operates as the genome of serovar Lai harbors the ATP-binding protein HlyB (LA0150) and HlyD (LA3737) on chromosome I (CI). The HlyB has a trans-membrane domain (TMD) associated with a nucleotide binding domain (NBD) (Benabdelhak *et al*., 2003) carrying a number of conserved distinct motifs, including the Walker A (GGSGVGKST; 412 – 420 residues), B (ILLLD) and the C-loop / signature motif (consensus sequence LSGGERQ; 516 – 522 residues) which is considered the hall mark of ABC transporter and the D-loop (consensus sequence SSLD; 544 – 547 residues), similar to the proposed conserved residues of *E. coli* (Zaitseva *et al*., 2005). However, unlike the cistronic organization of the *hlyCABD* operon responsible for hemolysin secretion seen in *E. coli* (Cross *et al*., 1990), the leptospiral *hlyB* and *hlyD* are not organized as an operon. Similarly, in *Neisseria meningitidis*, *hlyD* and *tolC* genes were adjacent but unlinked to *hlyB*, with the three genes being expressed independently (Wooldridge *et al*., 2005). In this study, the influence of iron levels on the iron acquisition machinery and the virulence factor sphingomyelinase has been explored. Much more remains to be understood in this aspect of host-pathogen interaction of these pathogens.

CHAPTER 5

CONCLUSIONS

5. Conclusions

In conclusion, we studied the influence of iron on the HbpA-mediated iron acquisition machinery and the virulence factor sphingomyelinase(s), specifically in *L. interrogans*. Incidentally we identified the outer membrane efflux protein, TolC[63] associated with sphingomyelinase transport. Conditions of iron-limitation were established for the growth of *Leptospira* spp. by the calculated addition of the iron chelator EDDA. The expression of HbpA was demonstrated in low-iron organisms of *L. interrogans* serovar Lai and could not be detected in serovars belonging to other leptospiral species. The surface expression of HbpA was shown by confocal microscopy. In addition to the iron-regulated hemin binding protein, another constitutively expressed 44 kDa protein was identified in outer membrane protein preparation; sequencing and immunoblotting with specific antibodies confirmed the protein to be the major lipoprotein LipL41.

Several lines of evidence showed that HbpA was restricted to serovars belonging to *L. interrogans* species. We extended this observation to assess the diagnostic potential of HbpA both by molecular (PCR) and immunological (ELISA) methods. The *hbpA* was amplified from all the 51 clinical isolates out of a total of 91 isolates from different geographical locations typed as *L. interrogans*. ELISA was encouraging with negligible cross-reactivity of the serum antibodies from patients with the purified rHbpA antigen.

Low-iron cultures of serovar Lai expressed a 42 kDa sphingomyelinase protein in the outer membrane vesicles (OMVs). Interestingly, the OMVs contained HbpA and LipL32. Notable was the absence of all these three proteins in the saprophytic *L. biflexa* serovar Andamana. The presence of sphingomyelinase, HbpA and LipL32 (hemolysis associated protein-1; hap-1) led us to hypothesis that a concerted action of these three pathogen-specific proteins in effective lysis of host cells.

We also identified a 63 kDa outer membrane protein in *L. interrogans* serovar Lai that was characterized as TolC, an outer membrane efflux protein (LA0957). It is not clear as to why this protein is recognized by anti-Sph antibodies, though it has been reported in *E. coli* that the toxin can still be associated on the cell surface. It is thus hypothesized that the 63 kDa protein possibly acts to transport the sphingomyelinase by a mechanism similar to HlyA of *E. coli*. Though the other components namely *hlyB* and

hlyD were identified in the genome, wet lab experiments are required to understand their role in the assembly of the multi-component system characteristic of the Type I secretion system seen in *E. coli*.

CHAPTER 6

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PUBLICATIONS

7. Research publications that has resulted from this study

1. Asuthkar S, **Velineni S,** Stadlmann J, Altmann F. & Sritharan M. 2007. Expression and characterization of an iron-regulated hemin-binding protein, HbpA from *Leptospira interrogans* serovar Lai. ***Infection and Immunity***. **75** (9): 4582 – 4591.

2. **Velineni S**, Manjulata Devi S, Ahmed N. & Sritharan M. 2008. Diagnostic potential of an iron-regulated hemin binding protein HbpA that is widely conserved in *Leptospira interrogans*. ***Infection, Genetics and Evolution*. 8:** 772 – 776.

3. **Velineni S**, Asuthkar S, Lakshmi V, Umabala P. & Sritharan M. 2006. Serological evaluation of leptospirosis in Hyderabad, Andhra Pradesh – a retrospective hospital based study. ***Indian journal of Medical Microbiology*. 25** (1): 24 – 27.

4. **Velineni S**, Asuthkar S. & Sritharan M. 2006. Iron limitation and expression of immunoreactive outer membrane proteins (OMPs) in *Leptospira interrogans* serovar Icterohaemorrhagiae strain Lai. ***Indian journal of Medical Microbiology*. 24** (4): 339 – 342.

5. Sritharan M, Asuthkar S. & **Sridhar V.** 2006. Understanding iron acquisition by pathogenic leptospires – a review. ***Indian journal of Medical Microbiology*. 24** (4): 311 – 316.

6. **Velineni S,** Rama Devi S, Asuthkar S. & Sritharan, M. 2008. Release of iron-regulated sphingomyelinase in outer membrane vesicles of *Leptospira interrogans* serovar Lai and the identification of $TolC^{63}$, an outer membrane efflux protein **(under communication).**