Iron limitation in *Leptospira* spp.: studies on a hemin-binding outer membrane receptor protein and the virulence factor sphingomyelinase

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the work embodied in this thesis entitled *"**Iron limitation in Leptospira spp.: studies on a hemin-binding outer membrane receptor protein and the virulence factor sphingomyelinase"*** is the result of the investigation carried out by me in the Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, under the supervision of **Dr. 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.

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CERTIFICATE

Certified that the work embodied in this thesis entitled "***Iron limitation in Leptospira spp.: studies on a hemin-binding outer membrane receptor protein and the virulence factor sphingomyelinase***" has been carried out by Ms. Swapna Asuthkar under my supervision and the same has not been submitted elsewhere for a degree.

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Abbreviations

ALP:	alkaline phosphatase
BCA:	bicinchoninic Acid
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
BLAST:	Basic Local Alignment Search Tool
Bp:	base pair
BPROM:	Bacterial Promoter analysis software
CAS:	Chrome Azurol S
CDS:	coding sequences
BSA:	bovine serum albumin
Da:	dalton
DNA:	deoxyribonucleic Acid
RNA:	ribonucleic acid
EDTA:	ethylene diamine tetra acetic acid
EDDA:	ethylenediamine-N, N'-diacetic acid
EDDHPA:	ethylenediamine di-o-hydroxyphenylacetic acid
ELISA:	Enzyme linked immuno sorbent assay
EMJH:	Ellinghausen & McCullough, modified by Johnson & Harris
FepA:	ferric enterobactin receptor protein
Fur:	ferric uptake regulator
H:	hour
HDTMA:	hexadecyltrimethyl ammonium bromide
IPTG:	isopropyl β-D-thiogalactopyranoside
IRPs:	iron-regulated proteins
Kan^r:	kanamycin resistance
Kb:	kilobase pair
KDa:	kilodalton
KV:	kilo volt
LhbpA:	leptospiral hemin binding protein A
LPS:	lipopolysaccharides
MAT:	Microscopic Agglutination Test
MEGA:	Molecular Evolutionary Genetics Analysis
µM:	micro molar
mg:	milligram
ml:	milliliter
mM:	millimolar
NBT:	nitro blue tetrazolium
NCBI:	National Center for Biotechnology Information
nm:	nanometers
OM:	outer membrane
OMP:	outer membrane protein
ORF:	open reading frame
PBS:	phosphate buffer saline
PCR:	polymerase chain reaction
Sph:	sphingomyelinase
SDS-PAGE:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TBS:	tris buffered saline
Tris:	tris - (Hydroxymethyl) aminoethane
TE:	tris-EDTA buffer
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 230 different serovars, whose distribution may be restricted geographically. The immense serovar diversity among pathogenic leptospires has been attributed to differences in the structure and composition of lipopolysaccharide. Despite the high antigenicity of these surface molecules, they afford little cross-protection against infectivity by other serovars. There is thus a need to identify new and novel antigenic determinants for effective control measures.

This can be achieved by a better understanding of host-pathogen interactions. Several factors contribute to host-pathogen interactions and the delicate balance between them determines the outcome of an infection. Acquisition of iron is one important contributing factor as limiting the amount of available iron is one of the mechanisms of control by the mammalian host Microorganisms have adapted themselves by the induction of novel pathways. They do so either by the expression of siderophores and their receptors or express surface receptors for direct acquisition from the host iron-containing proteins. Iron levels regulate the expression of the components of the iron acquisition machinery at the molecular level by interaction with a specific regulator protein, namely the Fur protein in *Escherichia coli* and several Gram-negative bacteria and the DtxR homologue in *Corynebacterium diphtheriae* and other Gram-positive bacteria. Interestingly, the iron-regulator complex controls, in addition, the expression of virulence determinants in several bacterial systems.

Though *Leptospira* require iron, mechanism of iron uptake is not known. In this study, we established the growth of *Leptospira* under high and low iron conditions. In *Leptospira biflexa* serovar Patoc strain Patoc I, we

demonstrated the expression of siderophores using the Universal Chrome Azurol S assay (CAS) and report the expression of four iron-regulated proteins of apparent molecular mass of 82, 64, 60 and 33 kDa respectively. Pathogenic *Leptospira* do not produce siderophores. Due to the difficulty in the establishment of iron limitation for pathogenic *Leptospira,* a bioinformatic approach was adopted to identify putative iron-regulated protein(s).

Homology search of the genome of *Leptospira interrogans* serovar Lai. with the ferric enterochelin receptor FepA of *E. coli* identified a TonB-dependent outer membrane receptor protein (LB191). This protein, modeled with the Insight II software showed a fold similar to ferric-siderophore receptors FepA, FhuA and FecA of *E. coli*. It showed the characteristic β-barrel domain, plug domain and the N-terminal TonB box seen in TonB-dependant outer membrane receptor proteins. The presence of the neighbouring *fur* gene (LB183) and a putative Fur box as well as LB186, encoding heme oxygenase and the FRAP/NPNL motif in the protein led us to hypothesise that this protein is an iron-regulated hemin-binding protein. We named this protein as LhbpA (leptospiral hemin-binding protein A).

To provide experimental evidence for the hypothesis, the full-length *lhbpA* gene was cloned and expressed from the serovar Lai. The hemin-binding ability of rLhbpA was analysed by three independent experiments. They included affinity binding to hemin-agarose beads and assay of the bound hemin, both spectrophotometrically and spectrofluorimetrically. In the latter, the quenching of the emission spectrum, with maximal intensity at 388 nm and the spectral shift to a lower wavelength indicated that hemin bound a specific site on the protein, possibly involving a tryptophan residue. The surface expression of LhbpA was confirmed by agglutination studies. Organisms, maintained in the presence of added chelators and maintained at 37°C for a few hours before harvesting, showed considerable agglutination in the presence of anti-LhbpA antibodies, as compared to the negligible effect in the presence of pre-immune serum; high iron organisms were unaffected by the

antibodies, implying the lack of expression of the surface LhbpA receptor. It was also observed that *lhbpA* was seen only in serovars of *L. interrogans*, though analysis of further serovars is needed to confirm this observation.

In the second half of the study, the leptospiral hemolysins, specifically the sphingomyelinases were analysed, with particular emphasis on the role of iron on their expression. A 638 bp DNA fragment (sph_{638}), common to the 4 sphingomyelinases *sph1*, *sph2*, *sph3* and *sphH* was chosen by ClustalW analysis, cloned and expressed in pET(28)a vector system. Using RT-PCR with specific primers, the sph_{638} transcript was demonstrated in organisms grown under low iron conditions. Low level of expression was however seen in high iron cells, possibly because one or more of the *sph* genes may be expressed constitutively. The *in vivo* relevance of this study comes from the detection of anti-Sph antibodies in the serum of patients with leptospirosis.

The study has given important insights into iron acquisition and role of iron in hemolysin expression in *Leptospira* spp. and has paved the way for further studies.

Review of Literature

1. Introduction

Leptospirosis is a zoonotic disease of global significance. The causative organisms are the pathogenic spirochetes belonging to the genus *Leptospira* (Bharti *et al*., 2003; Levett, 2001). In 1886, the human form of the disease was first described by Professor Adolf Weil of Heidelberg, who described it as a disease that was different from other known infectious diseases that caused jaundice and renal failure in humans. Today, the severe form of this disease is referred to as Weil's disease.

In 1907, Stimson demonstrated by silver staining the presence of clumps of spirochetes in the renal tubules of a patient, who was diagnosed as suffering from "yellow fever". He called them as *Spirocheta interrogans*, because the hook at the ends resembled a question mark. Later, the saprophytic organism found in fresh water was described by Wolbach & Binger (1914) and was named *Spirocheta biflexa*. Noguchi (1918) proposed the name *Leptospira,* meaning thin spirals after his detailed microscopic and culture observations. The disease, largely underreported till recently, is emerging as an important public health problem, with increase in the number of reported cases and outbreaks during the last decade.

1. 1. Features of the causative organisms

1. 1. 1. Taxonomy and Classification

Leptospires belong to the Order Spirochetales, Family Leptospiraceae and Genus *Leptospira*. The classification of *Leptospira* into different species is still not clearly defined, though two methods of classification prevail, the serological and

molecular methods, both of which are in use. The former was established initially, based on their antigen relatedness. The genus *Leptospira* was divided into two species: *Leptospira interrogans* that comprised of all the pathogenic leptospires found in animals and humans and *Leptospira biflexa* consisting of saprophytic leptospires found in the environment (later both the groups were called as *L. interrogans* and *L. biflexa* sensu lato). These two species were divided into several serovars based on cross-agglutination and cross-agglutinin-absorption tests (CAAT). The ***serovar*** is the basic taxon of *Leptospira* in the antigenic classification, with each serovar being represented by a reference strain. For practical reasons of diagnosis and epidemiology, serovars that show cross-agglutination with high titres with each other's antisera were grouped together into ***serogroups*** (Dikken & Kmety, 1978). More than 60 serovars of *L. biflexa* sensu lato and more than 200 serovars, arranged into 24 serogroups, are recognized within *L interrogans* sensu lato (Faine *et al*., 1999) with new ones being added as and when discovered.

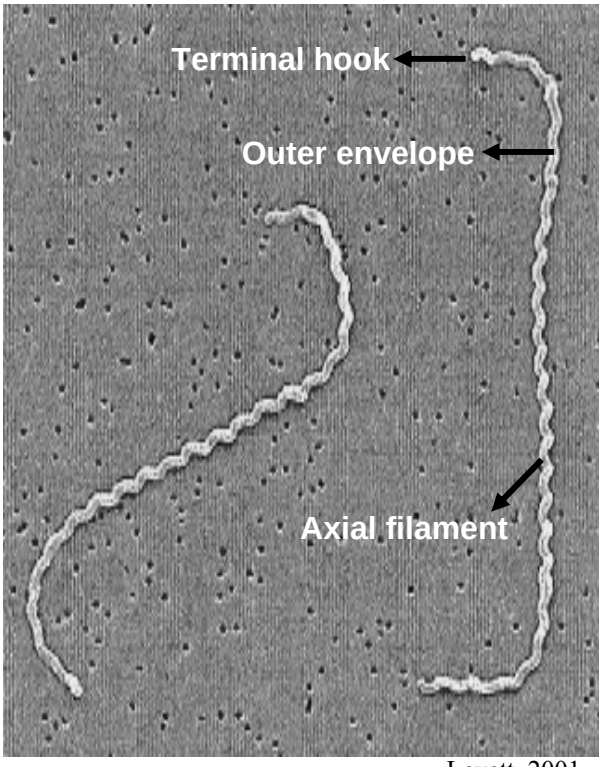
Molecular techniques showed that antigenically similar, perhaps even indistinguishable serovars might have highly variable genetic composition. These observations paved the way for molecular methods of identification of serovars (Brenner *et al*., 1999; Perolat *et al*., 1998; Ramadass *et al*., 1990; Ramadass *et al*., 1992; Yasuda *et al*., 1987) and today, the genetic classification, based on DNA hybridization has resulted in the division of leptospires into several genomospecies that include *L. alexanderi, L. alstoni, L. biflexa, L. borgpetersenii, L. fainei, L. inadai, L. interrogans, L. kirschneri, L. meyeri, L. noguchii, L. parva, L. santarosai, L. weilii*

and *L. wolbachii*. Newer methods, including analysis of the leptospiral DNA fragment length using restriction enzymes (REA) has proven to be useful for typing. Although the genetic classification has replaced the antigenic classification, the latter is still used for epidemiological analysis (Ellis, 1995). Novel methods are being developed, both by antigenic or genetic analysis with the purpose of establishing techniques that are less cumbersome and time-consuming for routine use.

1. 1. 2. Morphological features Leptospires are Gram-negative, obligate aerobes, helically coiled spirochetes with a length of 6-20 μm and a width of 0.1-0.15 μm (Levett, 2001) (Fig. 1). By virtue of their narrow diameter and poor staining ability, the leptospires are not easily identified by traditional staining techniques; they are best visualized by dark-field or phase contrast microscopy. The free living and pathogenic leptospires are morphologically indistinguishable (Faine, 1982). They have a characteristic bend or hook at one or both the ends, which are clearly visible by the spinning motility of the bacterium that is caused by a periplasmic flagellum at each end (Faine, 1982).

1. 1. 3. Ultra structure: Using the electron microscopy, the ultra structural organization can be visualized. The cylindrical cell body (protoplasmic cylinder) of the bacteria is helically wound around an axistyle (0.01-0.02 μm in diameter) that is comprised of two axial filaments (modified flagellum) (Bharti *et al*., 2003) attached sub-terminally at the extremities of the cell cylinder, with their free ends directed

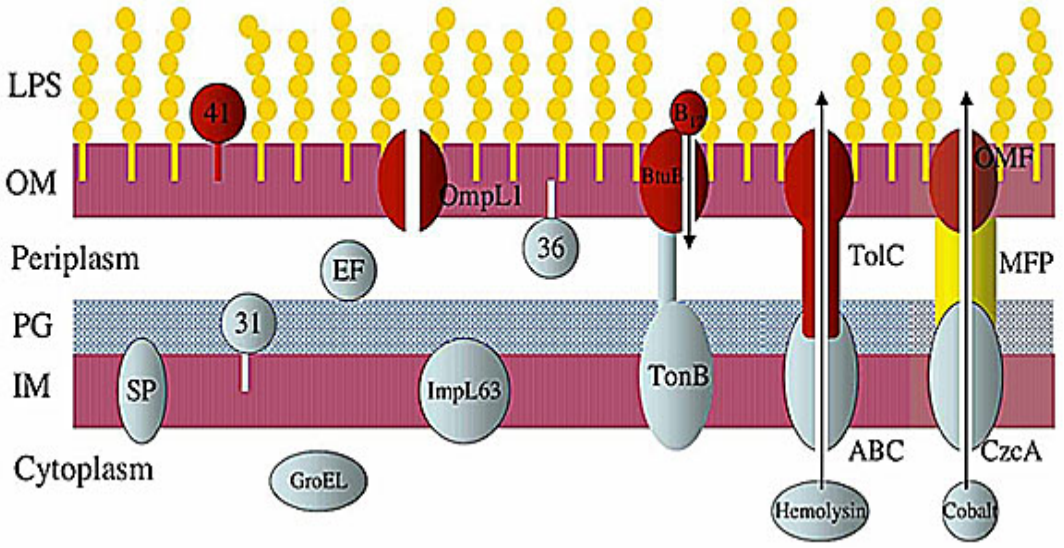
towards the middle of the cell (Charon & Goldstein, 2002). An outer membrane sheath envelops the axistyle and protoplasmic cylinder, which is demarcated by a cytoplasmic membrane. The space between the protoplasmic cell cylinder and the outer membrane sheath is referred to as the periplasm. The axial filament is thought to be a cytoskeletal element that enables movement of this highly invasive pathogen. The architecture of spirochaetes thus shares characteristics of both Gram-positive and Gram-negative bacteria. As in Gram-positive bacteria, the cytoplasmic membrane of spirochetes is closely associated with the peptidoglycan cell wall but has an outer membrane, which is a characteristic feature of Gram-negative bacteria (Fig. 2).



Levett, 2001.

Fig. 1 - Scanning electron micrograph of *L. interrogans* serovar Icterohaemorrhagiae strain RGA bound to a 0.2 µm membrane filter

1. 1. 4. Cell surface and outer membrane proteins. The cell surface of pathogenic *Leptospira* plays an important role in pathogenesis and immunity to infection due to its interaction with host cells.



Nascimento *et al.*, 2004

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 (PG) cell wall is closely associated with the IM. The leptospiral surface is dominated by lipopolysaccharide (LPS) carbohydrate side chains. Subsurface proteins include the cytoplasmic protein, GroEL, and the periplasmic endoflagella (EF). The IM contains lipoproteins such as LipL31 and transmembrane proteins such as signal peptidase (SP) and ImpL63. The OM contains lipoproteins including LipL41 and LipL36, and transmembrane proteins including the porin, OmpL1. Genomic sequence analysis reveals several novel types of outer membrane proteins (OMPs), including TonB-dependent OMPs involved in nutrient acquisition. BtuB is an example of a TonB-dependent OMP involved in uptake of vitamin B_{12}. The type I efflux system is represented by TolC, which forms a complex with ATP-binding cassette (ABC) transporters to export hemolysins and other cytoplasmic components. The leptospiral genome also contains genes involved in a three-component efflux system consisting of an outer membrane factor (OMF), membrane fusion protein (MFP), and an inner membrane transporter, in this case CzcA, which is involved in heavy metal detoxification.

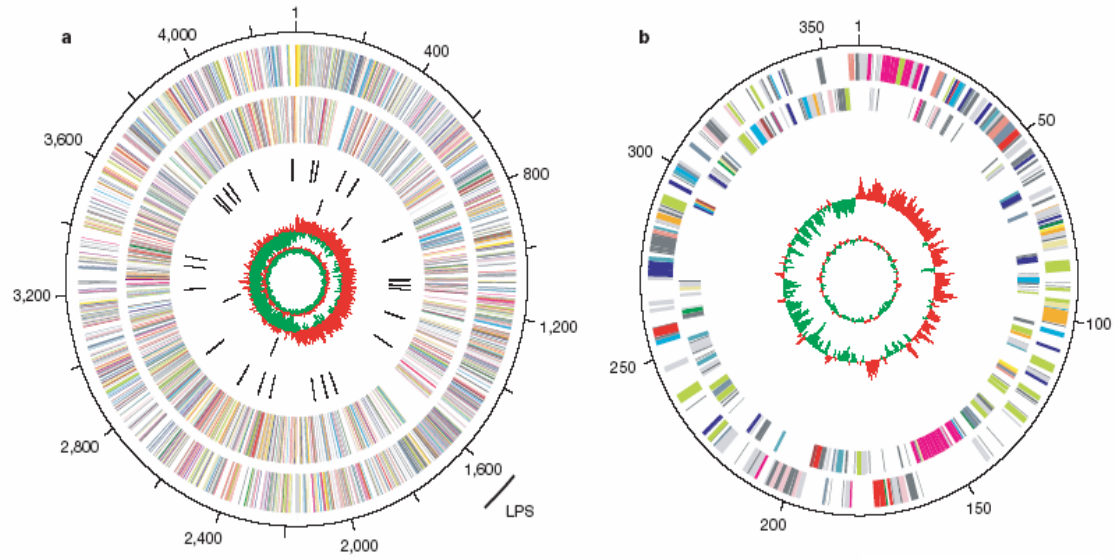
The leptospiral lipopolysaccharides form the major surface component of these organisms and contribute to the pathology of the disease. LPS is the target for agglutinating and opsonizing antibodies. The second major components of the leptospiral surface are the outer membrane proteins. Two types of leptospiral proteins have been characterized (Zuerner *et al*., 2000). The first type includes transmembrane OMPs, such as porins that traverse the outer membrane. OmpL1 is the first trans-membrane protein identified from a pathogenic spirochete (Haake *et al*., 1993). Q8F8Q0 is the second integral outer membrane protein reported by Cullen *et al*. (2005). The second type comprise the outer membrane lipoproteins, which are anchored to the periplasmic side of the outer membrane or to the extracellular face of the outer membrane by means of fatty acids covalently linked to an amino terminal cysteine (Zuerner *et al*., 2000).

Global analysis of leptospiral outer membrane proteins from *L. interrogans* serovar Lai grown at 20, 30 and 37°C and in iron-depleted medium at 30°C was studied by Cullen *et al*. (2002), using two-dimensional gel electrophoresis and the differentially expressed proteins were further characterized by mass spectrometry. Two mass variants of LipL36 and pL50 were expressed at 20°C and significantly down regulated at 30°C but were not apparent at temperatures above 30°C or after iron depletion at 30°C. The expression of pL24 was shown to be significantly down regulated at 37°C and 20°C and was almost absent after iron depletion. The leptospiral major outer membrane protein LipL32 was observed to undergo substantial C-terminal cleavage under all conditions except iron depletion. About 21 cleavage products and

pI isoforms of LipL32 were found which accounted for about 75% of the visibly expressed protein. At 30 and 37°C, all mass and pI forms of this protein could be detected at similar levels but at 20°C, LipL32.26 was significantly down regulated. A single cleavage product for LipL41 (LipL41.28) was observed at 30°C. The bacterial cell surface proteins were re-investigated by Cullen *et al.* (2005), using procedure consisted of biotin labeling of viable leptospires, affinity capture of the biotinylated proteins, two-dimensional gel electrophoresis followed by mass spectrometry. Three major proteins on the cell surface included LipL32, LipL21 and LipL41. Expression of the surfaceome constituents remained unaltered when studied under different conditions, including temperature, presence of 10% fetal calf serum and 10% urine from a healthy donor. LipL48 and LipL36 are located in the inner leaflet of the outer membrane (Haake *et al*., 1998). The p31 fragment of LipL45 (Matsunaga *et al*., 2002), GroEL a chaperone, and FlaB1 a flagellar subunit protein are identified as membrane-associated proteins. A pair of large leptospiral bacterial-immunoglobulin-like outer membrane proteins termed LigA and LigB were expressed only under *in vivo* conditions (Matsunaga *et al*., 2003) and are absent in the non-pathogenic *L. biflexa* serovar Patoc (Palaniappan *et al.*, 2004). These leptospiral immunoglobulin-like proteins are identical at the amino terminus but vary at the carboxyl terminus. In addition, the expression of the Lig proteins is dependent on osmolarity (Matsunaga *et al*., 2005).

1. 2. Genome analysis

The complete genomic sequence of *Leptospira interrogan*s serogroup Icterohaemorrhagiae serovar Lai type strain Lai (Ren *et al*., 2003), Copenhageni (Nascimento *et al*., 2004) and *L. borgpetersenii* serovar Hardjo (Bulach *et al*., 2006) is 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 4,768 predicted genes (Fig. 3). Later re-annotation (Bulach *et al*., 2006) of the serovar Lai resulted in the removal of 1,206 putative CDS and addition of 52 previously unidentified CDS to the previous Lai annotation, making the number of recognized CDS to a total of 3,613. Similarly, the serovar Copenhageni contains 3,530 recognized CDS after removal of 287 CDS and addition of 91 CDS. Both these genomes differs extensively from other related spirochetes, *Treponema pallidum* and *Borrelia burgdorferi*, and is much larger than these two spirochetes. Between the two genomes of serovars Lai and Copenhageni it was observed there was overall genetic similarity; however, 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.



Ren *et al*., 2003

Fig. 3 - Circular representation of the *L. interrogans* strain Lai genome, with predicted CDSs. Panel (a) represents large chromosome (CI) and Panel (b) represents small chromosome (CII). The outer scale is shown in kilo bases. Circles range from 1 (outer circle) to 6 (inner circle) for CI and from I (outer circle) to IV (inner circle) for CII. Circles 1/I and 2/II, genes on forward and reverse strand; circles 3, tRNA genes; circle 4, rRNA genes; circle 5/III, GC bias ((G-C)/(G+C); red indicates values>0; green indicates values<0; circles 6/IV, G+C content. All genes are colour-coded according to functions: orange for amino acid biosynthesis, green for purines, pyrimidines, nucleosides and nucleotides, blue for fatty acid and phospholipid metabolism, magenta for biosynthesis of cofactors, prosthetic groups and carriers, khaki for central intermediary metabolism, cyan for energy metabolism, orchid for transport and binding proteins, yellow for DNA metabolism, dark green for transcription, brown for protein synthesis, red for protein fate (protein and peptide secretion and trafficking), green–yellow for regulatory functions, pink for cell envelope, salmon for cellular processes, navy for other categories, light grey for conserved, dim grey for hypothetical, slate grey for unknown function protein, and black for tRNA and rRNA.

The recently sequenced genomes of *L. borgpetersenii* serovars Hardjo, L550 and JB197 (Bulach *et al*., 2006) show that the *L. borgpetersenii* genome is 16% smaller than *L. interrogans*. It is approximately 700 Kb smaller and has a lower

coding density than *L. interrogans*, indicating it is decaying 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 230 distinct 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 a 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 *L. borgpetersenii* tolerance to nutrient deprivation, increasing host dependence relative to *L. interrogans*.

1. 3. Epidemiology

The incidence of leptospirosis in humans is higher in the tropics than in temperate regions and transmission occurs in both industrialized and developing countries. The disease is more prevalent during the monsoon seasons, when heavy rainfall aids in the widespread contamination of the environment (Bharti *et al*., 2003). Incidence rates are underestimated due to lack of awareness of the disease and lack of affordable and reliable diagnostic techniques for early diagnosis.

Animal and human leptospirosis is spread worldwide (with >500,000 human cases annually with mortality rate up to 23%), but with higher incidence in tropical countries such as Latin America, South East Asia including India, Thailand (Hartskeerl, 2006). In these places, the conditions favour the survival and transmission of the leptospires. They include flooding, presence of several animal species that serve as reservoirs of infection, suitable climate for the survival of the bacteria and socio-economic conditions that favour transmission of these pathogens (Faine, 1982).

The disease has been referred by different names in different countries based on the occupational groups involved and the nature of the disease presentation. For, example, it is called as "seven-day fever" in Japan, "Cane cutter's disease" in Australia, "rice field leptospirosis" in Indonesia and "Fort Bragg fever" in USA.

In endemic areas, the number of leptospirosis cases may peak during the rainy season and even may reach epidemic proportions in case of flooding (WHO Report, 2007). In recent years, outbreaks of leptospirosis associated with natural calamities such as floods and cyclones are being reported with increasing frequency. Outbreaks have been reported from Nicaragua in 1995 following floods, from Orissa (India) in 1999 following the Super-Cyclone and floods, from Mumbai (India) in 2000 and 2005 following heavy rainfall and flooding and on several occasions in countries like Philippines and Thailand (Sehgal, 2006).

In India, the disease is largely under-reported. Historically, Taylor & Goyle (1931), during their extensive survey of the disease outbreak in the Andaman Islands incidentally isolated *Leptospira andamans* and *L. grippotyphosa*. In 1960, in Bombay

city serological evidence of *L. icterohaemorrhagiae* and *canicola* were found in five cases of jaundice (Dalal, 1960). In 1966, in Delhi, *L. icterohaemorrhagiae, L. pomona* and *L. canicola* were reported in suspected cases of PUO (Pyrexia of Unknown Origin) and in patients with jaundice (Joseph and 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). In 1983, in Madras, the seroprevalence of leptospirosis in jaundiced patients was 18% and it was 24% in PUO cases (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 of the 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, with titres ranging from 1:160 to 1:6400 against different serovars by MAT (Venkataraman *et al*., 1991). Among 54 patients admitted to the Government General Hospital, Madras, during November and December 1990 to 1991 with symptoms suggestive of disease, MAT titres of 1:1600 and 1:800 occurred in 39 and 51 cases respectively (Muthusethupathi *et al*., 1995). An outbreak of acute febrile illness with hemorrhagic manifestations and pulmonary involvement occurred in Diglipur of North Andamans during October to November 1993; 66.7% of the victims had significant titres of antibodies against *Leptospira* (Sehgal *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 in

South India; the epidemic followed severe flooding of the Tamil Nadu District in the autumn of 1993; 37 / 46 patients (80 %) had leptospiral DNA and 33 / 46 patients (72 %) were positive by serological tests (Kathryn *et al*., 1998). Outbreaks of leptospirosis have been increasingly reported from 1990. However, 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).

1. 4. Transmission

Leptospirosis is a zoonotic disease, in which the animal hosts play an important role in the transmission (Levett, 2001). Wild, domestic and peri-domestic animals, like rodents, bats and squirrels serve as reservoir hosts. Rodents serve as important carriers of the pathogens, thus playing a significant role in transmission. The carrier animals shed leptospires in their urine, thus contaminating the surrounding environment. Humans are accidental hosts, acquiring the infection either directly or indirectly from the contaminated environment. The most frequent sources of infection are soil and surface water contaminated with animal urine. Many occupational and recreational hazards are thus associated with leptospirosis; people at risk include

farmers, sewer workers, veterinarians, dairy workers, rice and sugar cane field workers and those who swim or wade in contaminated waters.

Transmission usually occurs to new hosts, 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, or through an invasion of the placenta from the mother to the foetus at any stage of pregnancy in mammals (Faine, 1994).

1. 5. Clinical manifestations

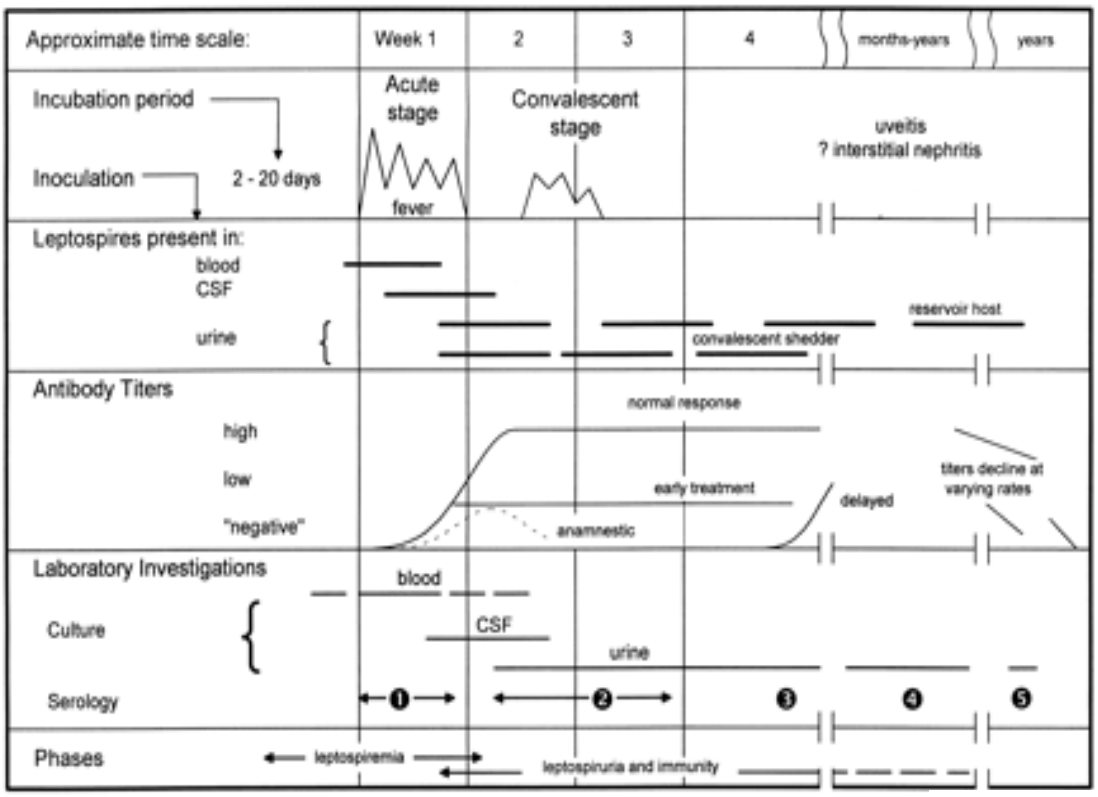
Leptospirosis has protean manifestations, and mimics the clinical presentations of many other diseases (Levett, 2001). Leptospirosis occurs as anicteric leptospirosis in 85% to 90% of the cases, with the severe form or Weil's disease in about 5 - 15% of cases, in whom it may prove to be fatal. The clinical presentation of leptospirosis is biphasic (Fig. 4), 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). Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and this occurs during the second week of the illness.

Leptospirosis manifests itself with a wide range of symptoms in humans, ranging from the mild flu-like syndrome to severe manifestations as in Weil's disease (Vinetz, 2001). In the former, the disease is usually self-limiting with symptoms that

includes sudden onset of high fever, severe headache, chills, muscle aches, vomiting, red eyes, abdominal pain, diarrhea or rash. The symptoms of this disease overlap with symptoms of flu, dengue and malaria and are thus difficult to diagnose clinically. Sub-clinical disease or symptom less infection is common and has been reported in several studies (Ashford *et al*., 2000). Pyrexia of unknown origin (Wood *et al*., 2004) includes leptospirosis and necessitates timely lab diagnosis of leptospirosis in order to treat the patient without delay. In the severe form of the disease, respiratory distress, pulmonary hemorrhage, renal damage, liver failure with severe jaundice may be seen that sometimes proves to be fatal (Vinetz *et al*., 1996). Leptospirosis should be considered in any case presenting with pulmonary haemorrhage and hepato-renal failure (Trevejo *et al*., 1998; Rao *et al*., 1998 and Darsun *et al*., 2007). Renal, cardiac, pulmonary and hemorrhagic complications occur in severe and icteric infections. In 1988 for the first time in India, post-monsoon outbreaks of febrile illness with haemorrhagic manifestations and high case-fatality rates have been reported in Andaman Islands. In 1993, Andaman haemorrhagic fever (AHF) was proved to be leptospirosis (Sehgal *et al*., 1995). In the 1997 outbreaks, pulmonary involvement has been the predominant complication, with haemoptysis as the common symptom. During the past few years, this form of presentation has also been observed occasionally in mainland India. In countries like China and Korea, the occurrence of pulmonary haemorrhage has been linked to infection with serovar Lai of serogroup Icterohaemorrhagiae (Oh *et al*., 1991). In Australia, pulmonary haemorrhage has been reported in patients infected with serovar Australis (Simpson *et al*., 1998). Serovar

Canicola of serogroup Canicola and serovar Pomona of serogroup Pomona were involved in the 1995 outbreak in Nicaragua (Zuerner & Bolin, 1997; Trevejo *et al*., 1998). Serogroup Canicola was also responsible for an outbreak of leptospirosis with pulmonary haemorrhage in Orissa, India, after a cyclone in 1999 (Sehgal *et al*., 2002). The identification of serovar Valbuzzi of serogroup Grippotyphosa was recently reported to be responsible for causing pulmonary haemorrhage (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. Prolonged oligouria may occur, the excretory functions are impaired, metabolic wastes accumulate and uraemic symptoms occur. The blood urea may exceed 70 mmol / L and serum creatinine may rise to 1.32 mmol / L. 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 seen both in the systemic bacteraemic phase as well as in the immunological phase. The incidence of ocular signs during acute systemic phase varies from 2% to 90%. 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).



Levett, 2001

Fig. 4 - Biphasic nature of leptospirosis and relevant investigations at different stages of disease.

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, hemorrhage (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 hemorrhagic syndrome of leptospirosis was studied in guinea pigs. The study correlates hematological, histopathological and immunohistochemical alterations in sixty animals inoculated by the intraperitoneal route with 1 ml of the culture of virulent strain of *Leptospira interrogans* serovar Copenhageni. Leptospiral antigens were detected by immunoperoxidase staining, chiefly in liver, kidney and heart muscle capillaries. Possible pathogenic mechanisms responsible for hemorrhagic 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). Leptospirosis may not be diagnosed among animals as they act as maintenance host that does not suffer or suffers mildly from the infection or sometimes the infection may run in the maintenance host as a chronic course with or without serious sequelae. No single diagnostic test for leptospirosis provides optimal sensitivity or specificity, and thus a combination of procedures, including serological tests to detect the presence of leptospires in tissues or body fluids, is recommended. In the developing countries due to limited laboratory facilities, the diagnosis becomes difficult and thus in these places the incidence, prevalence and economic importance of *Leptospira* infection needs to be further investigated (Nguyen, 1996).

1. 6. Host-immune response

The immunological response to leptospires is both humoral and cell-mediated, predominantly the former. The cell-mediated immune response occurs before the humoral immune response. After the entry of the organism into the host, both the B and T-cells are stimulated. The initial elimination of the bacteria is done by phagocytes. Opsonizing antibodies enhances the phagocytic activity of the polymorphonuclear cells, but do not reach the interior tissue such as renal tubules and Cerebrospinal fluid. The cell-mediated immunity induced by bovine and porcine vaccine gives good protection, which plays a major role in preventing renal localization (Levett, 2001 and 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. 4). High IgM levels can be observed during the first two months of the disease. Heterologous, i.e. genus-specific, antibodies appear first but decline faster; homologous, i.e. serovar-specific antibodies appear later and persist longer. 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. Fractionation studies demonstrated LipL32 and LipL41 reactivity in the outer membrane fraction, GroEL and DnaK in the

cytoplasmic fraction, p37 as a periplasmic protein and p48 and p45 as inner membrane proteins. The antibody response to lipopolysaccharide was predominantly IgM while antibodies to proteins were exclusively IgG. 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 in acute phase sera to be 30.2, 39.5, 27.9, 55.8 and 27.9% for the leptospiral proteins p32, p41/42, p58, p62 and p82 respectively. The IgG response considerably increased to 65.1, 55.8, 46.5, 67.4 and 48.8% against the same proteins during convalescent phase. The IgM recognition was 32.6, 32.6, 30.2 and 37.2% for acute phase sera and 32.6, 37.2, 44.2 and 41.9% for 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). The host-inducible LigA protein (Palaniappan *et al*., 2002) and sphingomyelinase-like protein, Lk73.5 (Artiushin *et al*., 2004) was isolated by screening the *L. interrogans* serovar Pomona genomic DNA library with serum from a mare that had recently aborted due to leptospiral infection.

1. 7. Control measures

The currently employed control measures are not adequate to control this disease. They should include early and definitive diagnosis of infection, effective

treatment and of greater importance preventive measures like vaccination of animals and humans aimed at blocking transmission.

1. 7. 1. Diagnosis

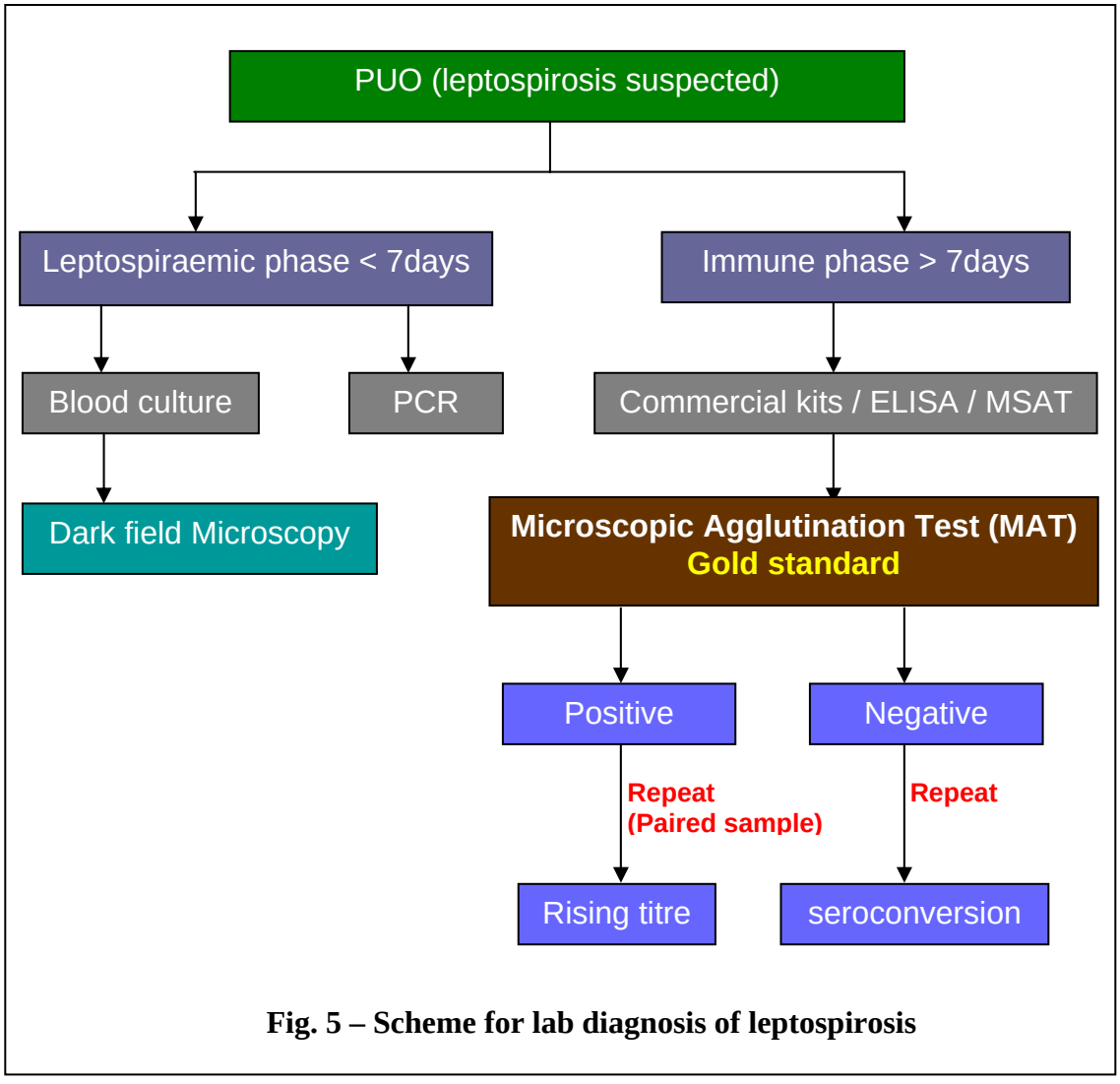


Fig. 5 – Scheme for lab diagnosis of leptospirosis

As in any bacterial disease, culture confirmation and identification by staining provides the definitive diagnosis of the infection. However, since leptospires are

fastidious and culture from blood and biological specimens is not easy, compounded by the difficulty in staining these organisms, serological methods are the preferred methods of detection. Due to the strong humoral immunity induced in response to infection with leptospires, antibody-detection based tests are used. When an individual presents with symptoms that is classified as PUO, leptospirosis is one of the suspected infections and the scheme of tests for the diagnosis of the disease is shown in Fig. 5.

1. 7. 1. 1. Direct demonstration of leptospires - Culture and dark field microscopy (DFM)

The isolation of leptospires by culture depends on the stage of the disease. Demonstration of leptospires is possible during the leptospiraemic phase, from day 1 - 10 after the onset of fever and initial symptoms. Blood culture (Wuthiekanun *et al.*, 2007) can be done during this period and preferably before antibiotics are given. Small inocula consisting of 1-3 drop of venous blood are inoculated at the bedside into 5 ml of media and incubated at 28-30°C for several weeks. The main disadvantage of blood culture is that it is slow and is not useful during epidemics. Direct demonstration by dark field microscopy (Faine, 1982), is being done for blood and urine samples, but suffers from the disadvantage of false diagnosis due to the presence of leptospiral-like artifacts in blood / serum samples. 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 remain renal excretors for more than a few weeks. Leptospires may be observed and

isolated from cerebrospinal fluid during the end of the first week of illness however, it is not usually performed. In fatal cases of human and animal leptospirosis, the organisms may be seen and cultured from ground post-mortem specimens liver, kidney and brain tissues (Brown *et al*., 2003).

1. 7. 1. 2. Serological methods

Leptospiral antibodies have been shown to appear in the circulation approximately one week after infection and the highest titres have been recorded ten days to three weeks after infection (Fennestad & Borg-Petersen, 1962). Thus, these methods are effective in diagnosis when serum is collected after a week upon onset of clinical symptoms. The most widely used test and recognized currently as the "Gold standard" for serological diagnosis is the Microscopic Agglutination Test (MAT). It is highly specific and sensitive and is due to the specific reactivity of the antibodies in the serum with the exposed LPS antigens (Cumberland *et al*., 1999). In this test, serum at varying dilutions are added to suspensions of live organisms, incubated for about three hours and visualized by dark field microscopy. The organisms 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 necessary to include several serovars, including the prevalent local isolates in this test.

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). 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; a four-fold rise in titre in MAT is then considered positive of active infection.

MAT has been useful in retrospective studies in confirming leptospirosis cases and identifying the prevalent serovar during that period. A retrospective hospital based study was done in our lab (Velineni *et al*. 2007) on 55 serum samples collected from suspected leptospirosis cases in Hyderabad, Andhra Pradesh. The samples were subjected to serological testing by Lepto Tek Dri-Dot, IgM ELISA and the results compared with MAT; all the samples were positive by MAT and the predominant serogroup identified was Icterohaemorrhagiae. Ismail *et al*. (2006), conducted a retrospective study to detect leptospiral antibodies in serum from patients with undiagnosed acute febrile illness (AFI) and hepatitis cases from Egypt and showed that approximately 16% of AFI (141/886) and acute hepatitis (63/392) cases showed seroreactivity by IgM ELISA and MAT. Canicola, Djasiman, Grippotyphosa, Pyrogenes, Icterohemorrhagiae and Pomona were the commonly reactive serovars among patients with AFI. Djasiman, Grippotyphosa and Icterohemorrhagiae were the most reactive among patients with acute hepatitis. A retrospective study of human

leptospirosis using MAT was done in Portugal and Azores islands for over a period of 18 and 12 years respectively, confirming leptospirosis as a public health problem in these places (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, considered as positive; thus MAT is of limited value in chronic infections and these low titres may remain for years after infection. Boqvist *et al*. (2002), collected a total of 424 sow's sera in the Mekong delta in Vietnam. Of these, 283 sows were from small-scale family farms and 141 from seven large-scale state farms. The sera, subjected to MAT analysis using 13 *Leptospira* serovars as antigens showed that the overall seroprevalence, in the small-scale and large-scale farms was 73 and 29%, respectively with titres ≥ 1:100 and ≥ 1:400 respectively. In the large-scale farms, the highest sero-prevalence was recorded for *L. interrogans* serovar Bratislava (52%), while in the small-scale farms, higher prevalences were found to *L. interrogan*s serovars Icterohaemorrhagiae and Pomona. 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, for dogs, against Icterohaemorrhagiae.

Enzyme-linked immunosorbent assay (ELISA) has practical applications in diagnosis of leptospirosis. As discussed earlier, due to the relatively higher levels of antibodies against leptospiral antigens in the population within an endemic area, suitable positive and negative control samples should be included in each assay. ELISA results are usually interpreted using a cut-off point, which is separately determined for each test in one's own laboratory. The cut-off point for the test sample is the antibody titre at which the absorbance is half the absorbance of the positive sample. Though the main advantage of ELISA, as compared to MAT is the stability of antigenic preparation, without a requirement for live organisms, ELISA results are normally correlated with MAT, as the former has lower specificity.

Antigenic preparations used in ELISA include cell-free whole cell sonicate, formalin-extract of a culture of leptospires and even whole leptospires coated on polysterene microtitre plates. Purified antigens and recombinant antigens have also been used in ELISA. Examples include the validation of outer membrane proteins like rLipL32 (Fernandes *et al.*, 2007) and immunoglobulin (Ig)-like Lig proteins (Croda *et al*., 2007) as antigens in ELISA. 92% of sera obtained from patients of acute-phase leptospirosis during urban outbreaks in Brazil have shown reactivity against the

recombinant fragment of LigB. IgM ELISA and MAT identified anti-LigB antibodies in sera from 57% of the patients who did not have detectable anti-whole-*Leptospira* response. The recombinant LipL41 antigen of *L. interrogans* serovar Canicola was used for serodiagnosis of bovine leptospirosis (Mariya *et al*., 2006).

Several commercial kits are now available in the market for diagnosis of leptospirosis. They include the PanBio ELISA kit (PanBio Pty Ltd., Brisbane, Australia), Serion ELISA kit (Institut Virion / Serion GmbH, Würzburg, Germany). The other antigen-antibody tests that are easier to use include the Lepto dipstick and LeptoTek Dri-dot (Organon Teknika in collaboration with the Royal Tropical Institute in Amsterdam), Latex agglutination test (Organon Teknika), a rLipL32 based test developed to detect specific anti-leptospiral antibodies from human and dog sera, and Microcapsule Agglutination Test (MCAT), based on the passive agglutination of synthetic polymer carriers sensitized with mixed antigens of sonicated leptospires, by leptospiral antibody. However, most of these kits need to be imported and are therefore not economically viable for many developing countries.

1. 7. 1. 3. Molecular methods

PCR has been used to demonstrate leptospires in tissues and body fluids. Hookey (1992) developed a specific PCR based on 16S rDNA that gave a product of 631 bp. Gravekamp *et al*. (1993) designed two sets of primers (G1 / G2) and (B64-I / B64-II) derived from genomic DNA libraries of *Leptospira* serovars Icterohaemorrhagiae and Bim respectively. Primers G1 (5'-CTG AAT CGC TGT

ATA ATA AAA GT) & G2 (5'-GCA GAA ATC AGA TGG ACG AT) enabled amplification of 285 bp DNA fragment from *L. interrogans, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai and L. meyeri*, whereas primers B64-I (5'-CTG AAT TCT CAT CTC AAC TC & B64-II (5'- GCA GAA ATC AGA TGG ACG AT) enabled the amplification of 563 bp DNA fragment from *L. kirschneri* in the serum samples collected from patients during the first 10 days after the onset of illness. Senthil Kumar *et al*. (2001), using specific primers for the amplification of a 330 bp fragment of 16S rRNA gene reported its application for the detection of leptospires in clinical samples. 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. Recently, a real-time PCR assay was developed by Levett *et al*. (2005), using a 423 bp target on the *lipL32* gene, conserved among the pathogenic serovars. Representative serovars from 16 species of *Leptospira* and over 40 species of other bacteria and fungi were tested. Positive results were obtained with all pathogenic leptospiral serovars, with the exception of *Leptospira fainei* serovar Hurstbridge. Magnetic Immuno PCR Assay (MIPA) developed by Taylor *et al*. (1997), is an improved technique on previously published PCR detection methods for the rapid detection of leptospires excreted in urine samples. The immuno-magnetic separation of leptospires from inhibitors in frozen formalin fixed bovine urine prior to PCR detection resulted in a marked improvement on previous detection methods. Kawabata *et al*. (2001), demonstrated that restriction fragment length

polymorphism (RFLP) of PCR products of *flaB* gene was an efficient tool for rapid detection and identification of species of infected *Leptospira* from clinical specimens.

Bolin *et al*. (1989) compared three techniques: nucleic acid hybridization, bacteriological culture and fluorescent antibody test for detection of *Leptospira interrogans* serovar Hardjo type Hardjo-bovis in bovine urine. They were able to detect organisms in 60 of 75 urine samples from cows by nucleic acid hybridization, 24 samples by fluorescent antibody test and 13 samples by bacteriologic culture. Ramadass & Marshall (1990) performed slot blot hybridization with total genomic DNA as probes to compare *L. interrogans* serovar Hardjo strain Hardjoprajitno and Hardjobovis and found that they different at the molecular level. Hence, they re-named Hardjobovis as *L. borgpetersenii* serovar Hardjo strain Bovis. Ramadass *et al.* (1997) characterized 14 laboratory strains of leptospiral serovars by RAPD finger printing and concluded this technique was rapid and sensitive and is a preferred method for serovar identification when compared to DNA restriction analysis. Natarajaseenivasn *et al*. (2004), combining serological testing with characterization of the isolates by RAPD finger printing showed the incidence of the disease in Erode, South India and indicated that the disease was a potential health hazard for agricultural workers in Cauvery basin.

1. 7. 2. Treatment

Though several cases of leptospirosis resolve spontaneously, treatment with pencillin and doxycycline is preferred when a definitive diagnosis of leptospirosis is

made. Doxycycline is recommended for both prophylaxis and mild disease (Takafuji *et al.*, 1984). Ampicillin and amoxicillin are also recommended in mild disease, whereas penicillin G and ampicillin are indicated for severe disease (Watt *et al.*, 1988). There are varying reports on the usefulness of administering doxycycline as a chemoprophylatic measure, but in general it appears to reduce rate of mortality and morbidity in endemic areas (Bharti *et al*., 2003).

1. 7. 3. Preventive measures

The objective of preventive measures should be to block transmission. They include vaccination, practice of proper hygiene and safe habits and maintenance of a clean environment. Vaccination strategies included the use of whole organisms, administered as formalin-treated bacterin preparations. The antigenicity was mainly due to the surface-exposed lipopolysaccharide (LPS). However, such vaccines were not effective as it was observed that the leptospiral LPS, though a protective immunogen generally does not induce long-term protection against infection and further does not provide cross-protective immunity against heterologous leptospiral serovars (Faine *et al*., 1999).

Animal experiments have shown that the outer membrane proteins are potential vaccine candidates. Combination of LipL41 and OmpL1 offered immunoprotection in hamsters, while neither protein was protective when administered alone (Haake *et al*., 1999). LipL32 delivered by recombinant adenovirus partially protected gerbils from acute infection (Branger *et al*., 2001). The two

immunogenic surface exposed lipoproteins, LigA and LigB induced protective immunity in mice. These proteins are of clinical significance as serum from patients with leptospirosis contained antibodies against these two proteins (Koizumi & Watanabe, 2004). The potential of rLigA as a vaccine candidate against infection by *L. interrogans* serovar Pomona in a hamster model was evaluated by Palaniappan *et al*. (2006). Golden Syrian hamsters were immunized at 3 and 6 weeks of age with rLigA using aluminum hydroxide as an adjuvant. Three weeks after the last vaccination, all animals were challenged intraperitoneally with 10^8 organism of *L. interrogans* serovar Pomona. All hamsters immunized with recombinant LigA survived after challenge and had no significant histopathological changes. In contrast, non-immunized hamsters that survived showed severe tubulo-interstitial nephritis. All vaccinated animals showed a rise in antibody titers against rLigA.

Currently, leptospiral vaccines are commercially available for animals. They include Leptavoid H (Scherring - Plough Animal Health, U.K) for cattle, Farrowsure (Pfizer Animal Health) for pigs and Nobivac (Intervet International) for dogs. Human vaccines are not used widely in Western countries. Immunization with polyvalent vaccines has been practiced in the Far East, where large numbers of cases occur in rice-field workers, as in China and Japan (Chen, 1985). A vaccine containing serovars Canicola, Icterohaemorrhagiae, and Pomona has been developed recently in Cuba (Sanchez *et al*., 1998). The Spirolept human vaccine, for vaccination against Icterohemorrhagiae was evaluated for immuno-protection in an experimental rodent

model (Rodriquez-Gonzalez *et al*., 2004). The immune human sera, administered into the experimental animals showed considerable protection in challenge studies.

Other preventive measures include personal hygienic and safe habits, such as the use of slippers / shoes and avoiding walking barefoot (especially by agricultural and sewer workers), preventing the contamination of the environment by maintenance of proper sewage disposal, control of rodent populations thereby reducing the rodent urine-mediated contamination of soil and water, maintenance of clean environment for domestic animals etc. Personnel involved in handling animals should regularly disinfect contaminated work areas, practice personal hygiene, and use protective equipment, like gloves and face shields when handling infected animals or tissues.

1. 8. Pathogenesis: an understanding of host-pathogen interactions

Host-pathogen inter-relationship influences the establishment of infection and the ability to cause disease. The establishment of infection depends upon the efficient and easy entry of pathogen into the host. The ability of pathogenic leptospires to cause rapid systemic infection after penetration of the skin suggests that it is a highly invasive pathogen. The characteristic helical morphology probably plays an important role in their rapid movement through the environment such as viscous gel-like connective tissues (Chunhao *et al*., 2000). Merien *et al*. (1997), observed that only virulent leptospires were internalized into both the non-phagocytic Vero cells and the phagocytic macrophage cell line. The ability of pathogenic strains and non-pathogenic strains of *Leptospira* to translocate through cellular barriers was shown to be

significantly faster in the former, suggesting its distinguishing feature of pathogenicity (Barocchi *et al*., 2002). Leptospires are invasive but are not facultative intracellular pathogens as they invade cells but escape from them rapidly to avoid intracellular killing (Barocchi *et al*., 2002). For *Leptospira* the ability to pass through cells may not be as important as the rate at which they penetrate them; rapid dissemination should be effected before cell barriers or circulating immune cells can inhibit or kill them.

Once the pathogen enters the host, it must adapt to the adverse microenvironments presented by the host in order to survive and establish an infection in the host. Adaptation mechanisms have been well studied in several bacterial systems. They have shown to regulate their gene expression in response to different environmental signals such as temperature, osmolarity, pH and nutrient limitation, especially iron deprivation (Salyers & Whitt, 1994). In addition, several pathogens express virulence determinants and specific gene products like toxins that enable them to counter the host defenses and establish themselves.

1. 8. 1. Temperature

Leptospires are routinely cultured *in vitro* at 30°C. However, as the pathogenic leptospires successfully establish and grow *in vivo* at 37°C within the mammalian host, several studies have been done on the influence of temperature on the expression of leptospiral proteins. Increased synthesis of 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 and Nally *et al*., 2001) was seen, with down

regulation of outer membrane lipoprotein LipL36 (Cullen *et al.*, 2002). The most abundant leptospiral surface protein, LipL32 was shown to undergo processing into a range of mass and pI isoforms, with the processing varying at different temperatures (Cullen *et al*., 2002). Whole genomic transcription analysis of leptospires maintained at 28°C and 37°C (Qin *et al*., 2006) showed the induction of about 106 genes belonging to nine functional categories. They include genes encoding hsp20 heat shock proteins, LA3927 encoding an outer membrane TolC protein that plays an important role in the secretion of extracellular hemolysins and enzymes, membrane biogenesis genes, intracellular trafficking and secretion genes, and information storage and processing genes. A similar study by Lo *et al*. (2006), by whole genome microarray hybridizations validated by real time RT-PCR, showed differential expression of chemotaxis and motility genes, signal transduction systems, and genes encoding transcriptional factors involved in alteration of the outer membrane.

1. 8. 2. Osmolarity

The shift in osmolarity to levels simulating that found in mammalian host tissues by the addition of sodium chloride, potassium chloride or sodium sulphate to the growth medium caused the induction of expression of LigA and LigB adhesins in pathogenic *Leptospira* (Matsunaga *et al*., 2005). Whole-genome microarray analysis of *L. interrogans* serovar Copenhageni, grown under different osmolar conditions (Matsunaga *et al*., 2007), showed that the transcript levels of 6% of the genes were significantly altered. They were predominantly the 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. The genome reduction seen in *L. borgpetersenii* serovar Hardjo, indicates it is transmitted directly between different hosts and appears to have lost genes necessary for survival outside the mammalian host.

1. 8. 3. Acquisition of iron

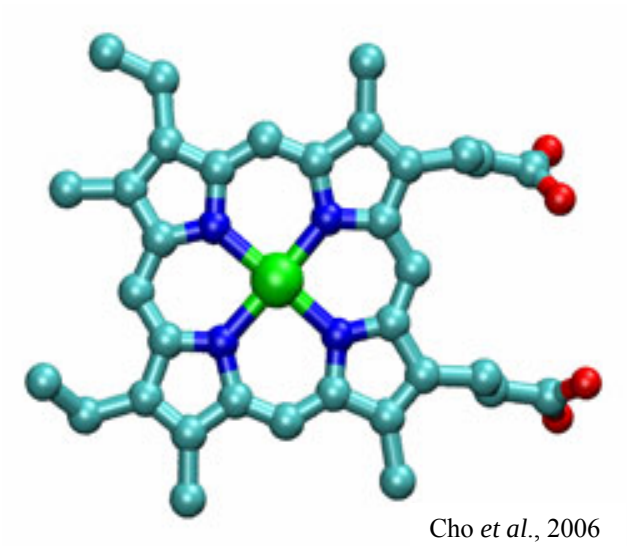
The acquisition of iron by a pathogen is recognized as one of the contributing factors in the successful establishment of the pathogen within the host. In the host-pathogen interaction, the balance between the ability of a mammalian host to withhold iron from invading microorganisms and the ease with 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). Bacterial pathogens have however adapted to conditions of iron limitation by the elaboration of novel iron acquisition machineries (Guerinot, 1994 and Payne, 1993).. Though iron is an essential element for pathogenic leptospires (Faine, 1982), little is known about the mechanisms of iron acquisition by these organisms. In this study, the focus is on understanding the iron acquisition machinery in leptospires. Hence a detailed review of bacterial iron acquisition mechanisms especially in *E. coli*, regulation at the molecular levels of not only the iron acquisition machinery but also the expression of virulence factors is detailed below

1. 9. Importance of iron and its bioavailability

Iron, by virtue of its ability to exist in both Fe^{2+} and Fe^{3+} states plays an important role in electron transport, DNA synthesis and as a cofactor for many enzymes (Griffiths & Chart, 1999 and Sritharan, 2000). Despite being the second most abundant metal after aluminium and the fourth most abundant element in the earth's crust, the insolubility of iron at biological pH renders this essential micronutrient a limiting factor for almost all bacteria. Iron in the inorganic environment exists as insoluble ferric hydroxides and oxy-hydroxides and is thus biologically unavailable. In the aerobic environment, at physiological pH 7, it has been recently shown that the major form of iron is $Fe(OH)_2^+$ (not $Fe(OH)_3$ as thought earlier) that has a solubility of approx 1.4×10^{-9} M (Chipperfield & Ratledge, 2000). The level of free iron is thus far less than the iron requirement of microorganisms, which is 10^{-7} M. Nature has perhaps made iron highly insoluble, as excess iron is highly toxic, due to its catalytic role in the formation of free radicals that is harmful for several macromolecules in biological systems (Sritharan, 2000). In addition, iron is held as protein-bound iron within the mammalian host; level of free iron is low as it is bound to transferrin and lactoferrin in the extracellular fluids and to ferritin intracellularly (Andrews *et al*., 2003). The former two iron-binding proteins are glycoproteins with high binding constants for the metal. Lactoferrin, present in monocytes and macrophages, the first lines of defense against invading pathogens can hold Fe^{3+} even within the acidic environment presented by these cells of defense. Ferritins, the primary iron storage

compounds of most organisms, composed of 24 protein subunits that form a hollow sphere can accommodate > 4000 atoms of Fe^{3+} as a macro-organic complex.

Another important source of iron within the mammalian system is heme. Heme is a rich source of iron. It is present largely in hemoglobin, which is a globular protein with a quarternary structure built from two alpha and two beta subunits, to each of which is bound a heme subunit (Fig. 6).



Cho *et al*., 2006

Fig. 6 - Molecular structure of heme. In the heme molecule shown above, the iron atom is green and the atoms in the porphyrin ring are carbon (teal), nitrogen (dark blue), hydrogen (not shown) and oxygen (red). At the core of the molecule is the heterocyclic ring of porphyrin which holds the iron atom. The iron atom binds equally to all four nitrogen's in the center of the ring which lie in one plane.

2. 0. Bacterial adaptation: High affinity iron acquisition systems

Bacteria have adapted to conditions of iron limitation and have developed high affinity iron uptake systems capable of acquiring this element from inorganic sources and the protein-bound iron within the mammalian host. (Neilands, 1990 and Sritharan 2000)

Two iron-acquisition machineries are identified in bacteria, namely the siderophore-mediated system and direct acquisition. The former is more commonly found and consists of low molecular weight Fe^{3+}-specific ligands called siderophores and their receptors, the iron regulated membrane proteins (IRMPs). The other equally important strategy adopted specifically by pathogenic bacteria is the direct removal of the protein-bound iron by the elaboration of specific receptors for the host iron-containing molecules, transferrin, lactoferrin, hemin and hemoglobin.

2. 1. Siderophores and Iron-regulated membrane proteins

Siderophores are low molecular weight (500-1000 Da) Fe^{3+}-specific high-affinity molecules, whose binding affinity for iron (K_s) range from 10^{22} to 10^{50} and are thus capable of removing iron from molecules such as ferritin, transferrin, lactoferrin, but not from heme proteins. Siderophores and their receptors, the iron-regulated membrane proteins, extensively studied in *E. coli* (Neilands 1990 and Griffiths & Chart, 1999) are expressed by several bacteria, as listed in (Table 1).

Table 1-Bacterial siderophores and their receptors, the iron-regulated membrane proteins

Organism	Siderophores	Iron-regulated membrane proteins	
		Protein	**Molecular size (kDa)**
Escherichia coli	Ferrichrome	FhuA, Ferrichrome receptor (Coulton *et al*., 1983)	78
	Enterobactin	Fep A, Ferric enterobactin receptor (McIntosh & Earhart, 1977)	81
	Ferri citrate	FecA, Ferric dicitrate receptor (Wagegg & Braun, 1981)	80.5
	Aerobactin	CirA, Aerobactin receptor (Curtis *et al.*, 1988)	74
Pseudomonas aeruginosa	Pyochelin	Ferri-pyochelin receptor (Sokol & Woods 1983)	14
	Pyoverdin	Ferri Pyoverdin receptor (Meyer *et al*., 1990)	80
Yersinia enterocolitica	Yersiniabactin	FyuA, Yersiniabactin receptor (Rakin *et al*., 1994)	71.4
Vibrio cholerae	Vibriobactin	ViuA. Vibriobactin receptor (Butterton *et al*., 1992 and Stoebner *et al*., 1992)	74
Vibrio vulnificus	Vulnibactin	VuuA, Ferric vulnibactin receptor (Webster & Litwin, 2000)	72

In *E. coli*, about 6 siderophore-mediated iron-transport systems have been studied in considerable detail. FhuA, FepA and FecA are three of the well characterized 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 in the periplasmic side and longer ligand-specific external loops of varying sizes that extends above the cell surface. The β-barrel forms the central channel of the siderophore receptors, which is completely closed by a globular plug domain, derived from the 160 residues at the N-terminus and is positioned in the pore towards the periplasmic end of the barrel. These ferric siderophore receptors interact with the TonB protein via the TonB box present in its amino terminal end. The TonB protein is anchored in the cytoplasmic membrane by an uncleaved leader sequence and spans the periplasmic space to interact directly with the receptor in the outer membrane. The TonB box precedes the plug domain that closes the β-barrel. The TonB box comprises of residues 6-11 in FhuA, 11-16 in FepA, and 80-84 in FecA. The mechanism of uptake via these ferric siderophore receptors is discussed below.

2. 2. Direct uptake of iron from the host proteins: Receptors for Transferrin, Lactoferrin, Heme and Hemoglobin

The second important mechanism includes the direct acquisition of the protein-bound iron by elaborating specific cell surface receptor proteins for heme, heme proteins, transferrin and lactoferrin (Braun & Killmann, 1999 and Schryvers & Stojiljkovic, 1999).

Table 2- Direct acquisition of iron-Hemin / Hemoglobin / Transferrin / Lactoferrin receptors

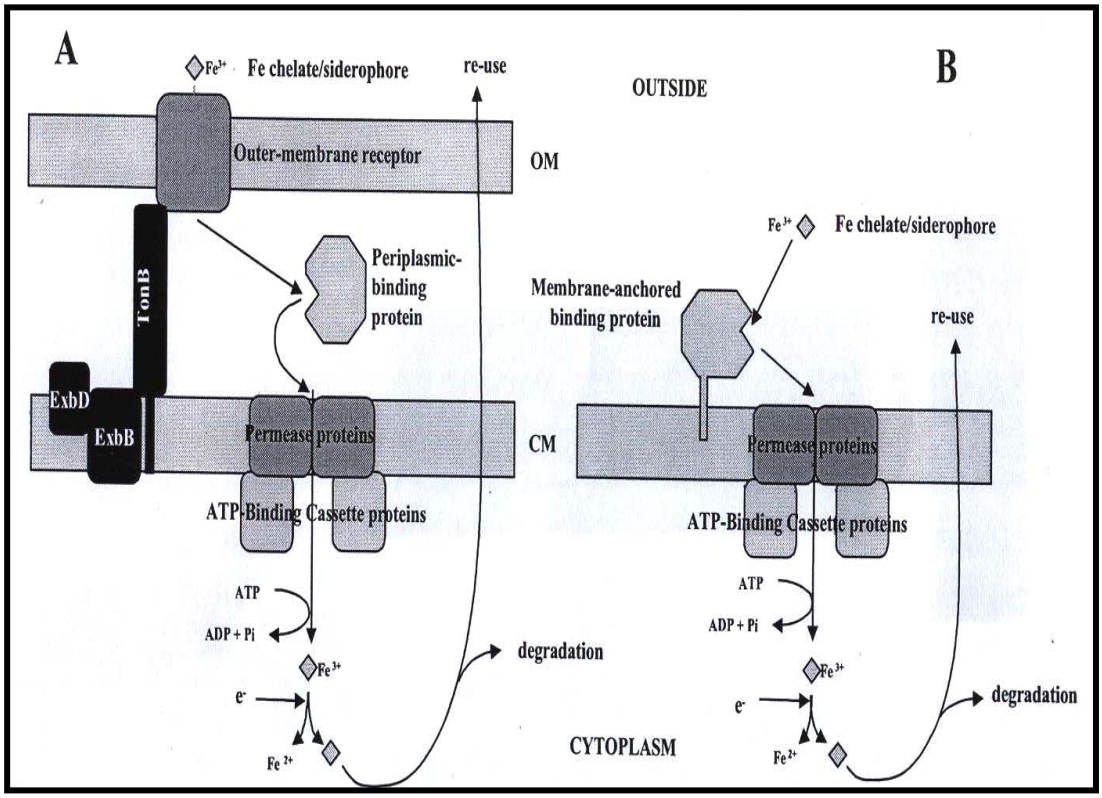
Organisms	Iron-regulated membrane proteins	
	Protein	Molecular size (kDa)
Vibrio cholerae	HutA, heme receptor (Henderson & Payne, 1994)	77
Vibrio vulnificus	HupA, heme receptor (Litwin & Byrne, 1998)	77
Vibrio parahaemolyticus	HupA, heme receptor (O'Malley *et al*., 1999) HupO (Ahn *et al*., 2005)	77 77
Vibrio fluvialis	HemR, heme receptor (Stojiljkovic & Hantke, 1992)	78
Yersinia enterocolitica	HxuA, heme-hemopexin receptor (Cope *et al*., 1994)	100
Haemophilus influenzae	Tbp1, Transferrin receptor Tb2, Transferin receptor (Gray-Owen *et al*., 1995) Heme-human hemopexin-binding protein (Hanson *et al*., 1992)	100 85
Serratia marcescens	HasR, heme receptor (Ghigo *et al*., 1997)	98
Treponema denticola	HbpA, hemin receptor (Chu *et al.,* 1994) Lactoferrin binding proteins (Staggs *et al*., 1994)	44 50 & 35
E. coli O157:H7	ChuA, hemin receptor (Torres & Payne, 1997)	69
Neisseria gonnorrhoeae	HpuB, Hemoglobin receptor (Chen *et al*., 1996) HmBP, hemin binding protein (Lee & Levesque, 1997) Tbp1, Transferrin receptor (McKenna *et al*., 1988) LbpA, Lactoferrin receptor (Biswas & Sparling, 1995) HmbR and HpuB, Hemoglobin receptor (Stojiljkovic *et al*., 1995 and Lewis & Dyer, 1995)	89 97 37 103 89.5 & 85
Neisseria mengitidis	Transferrin receptor (Schryvers & Morris, 1988)	71
Borrelia burgdorferi	Tbp, Transferrin binding protein (Caroll *et al*., 1996)	28
Staphylococcus aureus	Tbp, Transferrin binding protein (Modun *et al*., 1994)	42

This is well studied in members of *Pasteurellaceae* (Ogunnariwo & Schryvers, 1990) and *Neisseriaceae* (Genco & Desai, 1996). Heme is the largest potential source of iron within the mammalian host (Otto *et al*., 1992). Several bacteria elaborate hemin receptors for acquisition of not only iron but also heme, as many of them lack the ability to synthesize heme. Table 2 gives the transferrin, lactoferrin and hemin receptors in different bacterial systems.

2. 3. Mechanism of uptake by the ferric siderophore / hemin receptors.

2. 3. 1. Cell wall organisation in Gram-negative and Gram-positive bacteria

The cell wall of Gram-negative bacteria consists of an outer membrane (OM), which is separated from the inner cytoplasmic membrane (CM) by the periplasmic space, unlike the Gram-positive bacteria, which elaborates only the cytoplasmic membrane. In Gram-negative bacteria, the uptake of Fe^{3+} bound to siderophores and heme occurs via specific outer membrane receptors with the internalization mediated by the TonB protein (as described below), with the transport across the cytoplasmic membrane being mediated by an ABC transport system. In Gram-positive bacteria the uptake is by ABC transport system with the help of membrane-anchored binding proteins. This is represented in Fig. 7.



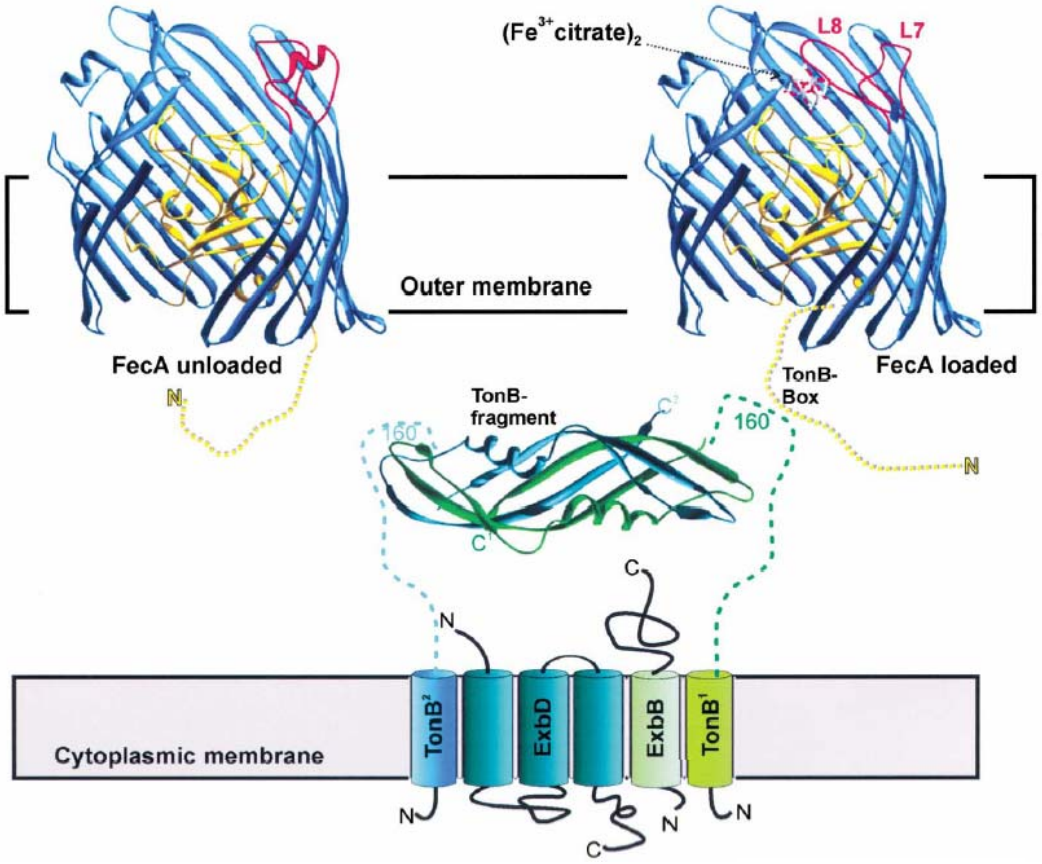
Andrews *et al*., 2003

Fig. 7 - Diagrammatic representation of iron uptake mechanisms in Gram-negative (A) and Gram-positive (B) bacteria.

2. 3. 2. Mechanism of transport in Gram-negative bacteria.

Ferric siderophore and hemin receptors, being TonB-dependant outer membrane proteins employ a similar mechanism for internalizing the ligand. Also, these iron transport receptors are multi-functional and mediate the transport of vitamin B12 and certain colicins. Gram-negative bacteria that can use Fe^{3+} bound to proteins, heme and siderophores contain highly specific transport proteins in their outer membranes.

Upon binding of the specific ligands, the receptor proteins undergo conformational change. Then, interaction with the TonB protein via its TonB box for the opening of the channel of the β-barrel by subtle rearrangements of the loops of the plug domain through which iron / ferric siderophore / hemin molecule is translocated to the inside. Transport of ferri-siderophores through these outer membrane receptors requires energy. This energy is provided by the electrochemical charge gradient of the cytoplasmic membrane and is delivered by the energy-transducing TonB-ExbB-ExbD protein complex working coordinately (Braun and Braun, 2002). ExbB and ExbD are integral CM proteins, whereas TonB is periplasmic and anchored to the CM 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 OM. TonB-ExbB-ExbD complex interact with each other, the reaction being mediated by the proton-motive force of cytoplasmic membrane, which serves as an energy source. 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 ferric siderophores (Fig. 8).



Braun & Braun, 2002

Fig. 8 -Transport of $(Fe^{3+}$-citrate$)_2$ across the outer membrane of *E. coli*. The crystal structures of the FecA outer membrane transport protein unloaded and loaded with $(Fe^{3+}$-citrate$)_2$ disclose the large structural transitions of surface loops 7 and 8 and of the region exposed to the periplasm. The crystal structure of a C-terminal dimeric fragment of TonB (residues 164-239) is shown on the same scale as FecA, which demonstrates that only one region 160 at a time is able to interact with the TonB box of FecA

The subsequent transport of siderophores into the cytoplasm is facilitated by periplasmic binding protein dependent transport (PBT) 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 ferri – reductases.

$$Fe^{3+}\text{siderophore} + NAD(P)H \longrightarrow Fe^{2+}\text{ siderophore} + NAD(P)^{+}$$

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 *Serratia marcescens*, involves the secretion of a soluble protein called as hemophore, that chelates the heme from heme-proteins and releases the heme at the cell surface; uptake of the heme occurs via a TonB-dependent receptor (Wandersman & Stojiljkovic, 2000 and Ghigo *et al*., 1997). The *S. marcescens* haemophore-dependent heme acquisition system consists of the iron-regulated *has* operon encoding HasR, the hemophore-specific outer membrane receptor, HasA, the hemophore, HasD and HasE, the specific inner membrane hemophore secretion proteins (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.

2. 4. Regulation by iron at the molecular level

The molecular mechanism of regulation of the iron acquisition machinery is well studied, with extensive information in *E. coli* (Braun *et al*., 1998; Sritharan, 2000). Iron operates at the molecular level in association with a regulator protein. In *E.*

coli and several Gram-negative organisms, the *fur* gene and its protein product, the Fur repressor play a key role in exerting control over the expression of many of the genes involved in the uptake of iron (therefore aptly referred to as Fur-ferric uptake regulatory protein). The Fur protein in *E. coli* is a 17 kDa molecule, which on complexing with Fe^{2+} blocks the transcription of iron-regulated genes by binding to specific sequences located within their promoter regions, called Fur box / Iron box, whose consensus sequence was determined as 5'- GATAATGATAATCATTATC -3'. When iron is present is adequate amounts, there is repression of the synthesis of the genes whose promoter is under the control of the Fur repressor. Under iron limitation conditions, the repressor molecule does not bind to the iron box resulting in the expression of siderophores and their transport proteins.

In Gram-positive bacteria, the DtxR repressor, first identified in *C. diphtheriae* is the Fur homologue. The Fur repressor in the Gram-negative bacteria and the DtxR in the Gram-positive bacteria control not only the transcription of the iron sequestering genes but exert a direct control over the expression of virulence determinants.

2. 5. 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 outer membrane proteins. LipL32, LipL36 and pL50 were some of the proteins that showed changes in their

expression under alterations of iron / temperature. 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 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). Genome analysis has shown that the *Leptospira* spp. possesses a complete heme biosynthetic pathway and is also capable of using exogenous heme sources (Guegan *et al*., 2003).

2. 6. Iron and virulence

A number of environmental signals like pH, osmolarity, levels of certain nutrients control the expression of virulence genes. Iron levels and expression of virulence factors are linked in many bacterial systems like *E. coli, C. diphtheriae, P. aeruginosa, V. cholerae* etc. (Salyers & Whitt, 1994). The synthesis of molecules needed for iron sequestration and the establishment of disease is well documented in many of these pathogens. Though the ability of pathogens to acquire iron from their hosts is an important contribution to the virulence of the organism, it cannot be said that the ability of a bacterium to acquire iron could render it virulent.

The relationship between iron and bacterial virulence has been studied in experimental animals. The virulence of the organisms and their multiplication was 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 *Y. enterocolitica* (Robins-Brown & Prpic, 1985), *S. aureus* (Gladstone & Walton, 1970) *V. cholerae* (Ford & Hayhoe, 1976), *V. vulnificus* (Wright *et al.,* 1981).

Iron controls the expression of virulence determinants, for example, diphtheria toxin produced by *C. diphtheriae*. This Gram-positive organism that colonizes in the throat produces this exotoxin, which is responsible for the damage and the disease symptoms. It is interesting to note that the production of the toxin is increased significantly when the bacteria are grown in low iron medium. At the molecular level, it is now proved that the 25 kDa repressor protein DtxR is unable to bind DNA in the absence of iron. When Fe^{2+} binds to it as a co-repressor molecule, DtxR-Fe binds to the –10 region of the promoter and the transcription start site of the *tox* gene thereby blocking its transcription by RNA polymerase. Thus under low iron conditions, the toxin production is increased. It has been proposed that the toxin, by virtue of its property of lysing the host cells, release iron from the iron stores within these cells. It is thus clear that the toxin production is part of the iron acquisition machinery of this pathogen.

The role of iron in other bacteria is exemplified by its influence on the expression of aerobactin by *E. coli*, exotoxin A by *P. aeruginosa*, anguibactin by *V.*

anguillarum, Shiga toxin by *Shigella* spp. and the virulence-associated iron-regulated protein *irg*A and the hemolysin in *V. cholerae* (Salyers & Whitt 1994). These pathogens express these iron-regulated toxins or virulence determinants to successfully establish infection in the host. Hemolysins are expressed by several bacteria including *V. fluvialis*, *S. marcescens, Porphyromonas gingivalis*, *Aeromonas caviae* and *Edwardsiella tarda* in whom their expression is linked with iron availability (Stoebner & Payne, 1988; Poole & Braun, 1998; Chu *et al*., 1991; Karunakaran & Devi, 1994 and Janda & Abbott, 1993). In the iron-deficient environment of the host, hemolysins act on erythrocytes and other cells, causing cell lysis that release iron or iron containing compounds to the outside. The spirochete *Treponema 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).

2. 7. Virulence determinants in *Leptospira* spp.

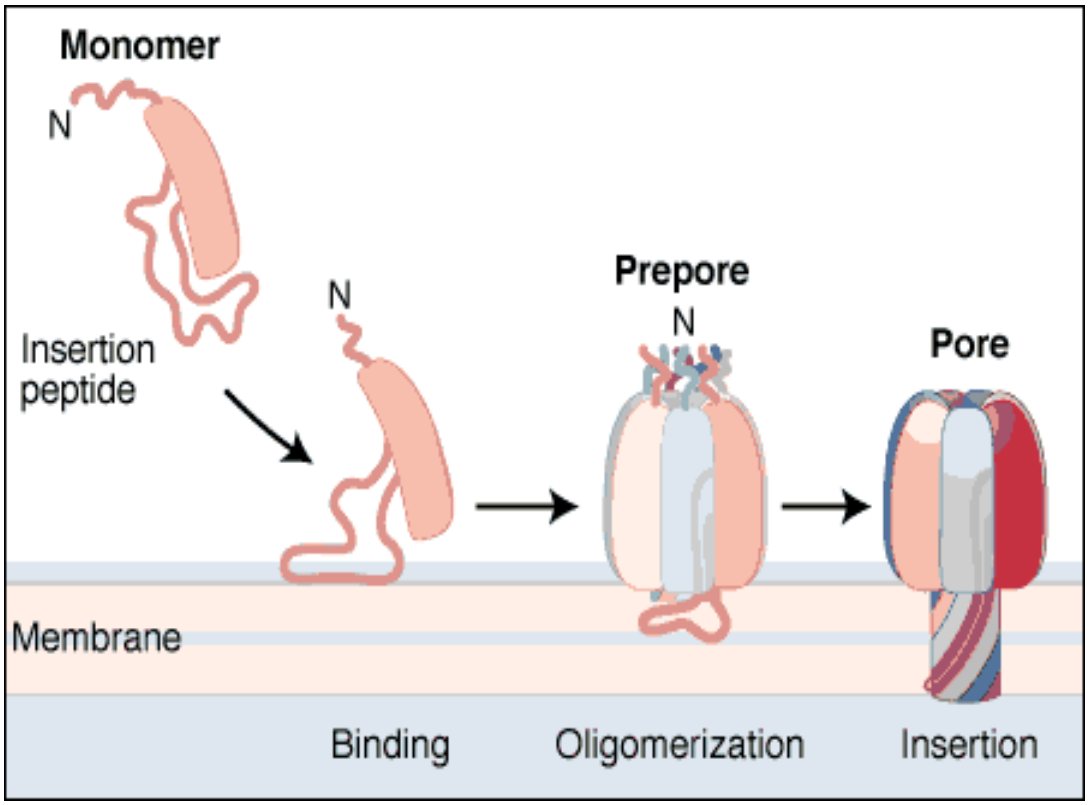
Based on the genome analysis, several virulence determinants of *Leptospira* have been (Ren *et al*., 2003, Nascimento *et al*., 2004). They include flagellin, adhesins, lipopolysaccharides, glycolipoproteins, peptidoglycan, heat shock proteins and hemolysins. About 50 genes are associated with motility, which may confer selective advantages in adapting to and migrating through host tissues and several proteins expressed during mammalian infection, for example the leptospiral

immunoglobulin like protein A (LigA) with attachment and invasion functions (Palaniappan *et al*., 2002).

2. 7. 1. Leptospiral hemolysins

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 *et al*., 1994). Hemolysins causing cytolysis by enzymatic disruption of target cell membranes include phospholipases such as α-toxin from *C. perfringens*, β-toxin (sphingomyelinase) of *S. aureus* and phospholipases from other Gram-positive or Gram-negative bacteria. These phopholipases have shown a high degree of substrate specificity and have reacted with multiple target cells. The delta toxin of *S. aureus* and the heat stable hemolysin from *P. aeruginosa* are surfactants. These toxins are highly hydrophobic and act like detergent, causing cytolysis by solubilization of target cell membrane.

The pore forming hemolysins function by binding to the surface of mammalian cells, inserting into the bilayer and creating holes in the membrane that leads to cell death. This group of toxins includes the RTX (repeats in toxin) toxins from Gram-negative bacteria, streptolysin O produced by *S. pyogenes*, and the *S. aureus* α-toxin.



http:// www.wellesley.edu

Fig. 9 - Pore formation by bacterial toxins.

S. aureus α-toxin can be considered the prototype of oligomerizing pore-forming cytotoxins. For α-toxin to damage cellular membranes, three sequential events are required (Fig. 9). Toxin protomers must first bind to target membranes by either high-affinity receptors or through non-specific absorption to substances such as phosphatidylcholine or cholesterol on the lipid bilayer. Second, membrane-bound protomers must oligomerize into a non-lytic pre-pore heptamer complex. Third, the heptamer must undergo a series of conformational changes that create the stem domain of the toxin, which is then inserted into the membrane. The α-toxin pore allows the influx and efflux of small molecules and ions by inserting a transmembrane

pore that eventually lead to the swelling and death of nucleated cells and the osmotic lysis of erythrocytes.

Alexander *et al.* first reported hemolysins in *Leptospira* in 1956. This was followed by several other reports (Alexander *et al*. 1971; Stamm & Charon, 1979; Bernheimer & Bey, 1986; Real *et al*., 1989; Segers *et al*., 1990, 1992 and Lee *et al*., 2002). Leptospiral hemolysins were found to be heat-labile, with complete loss of the hemolytic activity when heated at 56°C for 5 min. They are considered to be phospholipases, with phospholipase A and sphingomyelinase A activities demonstrated (Bernheimer and 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 and showed it to be homologous to sphingomyelinase genes from Gram-positive bacteria. Later, the same group, based on hybridizing DNA fragment of sphingomyelinase, which they referred to as putative sphingomyelinase genes (PSGs) showed they were present in all pathogenic members of *Leptospira* spp. and were notably absent in the saprophytic members (Segers *et al*., 1992).

While the hemolytic and sphingomyelinase activities were identified in the culture filtrates in most of the above reports, Segers *et al*. (1992), while demonstrating a similar finding with the serovar Pomona found that the sphingomyelinase activity of strains MUS127 (serovar Ballum) and Sponselee (serovar Hardjo) were cell associated (Segers *et al*., 1990). Lee *et al*. (2002) cloned and characterized *sphH*. They

demonstrated that SphH is a pore-forming hemolysin and lacks both sphingomyelinase and phospholipase activities. Using transmission electron microscopy, they demonstrated the membrane-disrupting effect of SphH on mammalian cell lines. Recently, Hauk *et al*. (2005) studied the hemolytic activity of HlyX hemolysin and showed its effect and the potentiating effect of LipL32 on hemolysis. The latter, as discussed earlier is an outer membrane protein that potentiates hemolysis and its referred to as hemolysis-associated protein (Hap-1).

Hemorrhage is commonly seen in the severe form of leptospirosis, involving the lungs, liver and kidneys resulting more often in death. The hemolysins, playing an important role in the pathogenesis of this disease thus need to be evaluated as vaccine candidates. With the genomes of *L. interrogans* (serovars Lai and Copenhageni) and *L. borgpetersenii* being delineated (Ren *et al*., 2003; Nascimento *et al*., 2004 and Bulach *et al*., 2006), the information provides an important opportunity for functional genomic studies. Using reverse vaccinology, potential vaccine candidates were identified for *Neisseria meningitides* (Pizza *et al*., 2000) and *Streptococcus pneumoniae* (Wizemann *et al*., 2001). Similar strategy may be adopted in the case of pathogenic *Leptospira*, especially *L. interrogans*.

Objectives of the study

I. Iron acquisition in *Leptospira* spp.

1. Establish low and high iron conditions for the growth of *Leptospira* spp.
2. Studies on the saprophytic *Leptospira biflexa* serovar Patoc strain Patoc I:
    - Assay for the expression of siderophores
    - Identification of iron-regulated proteins
3. Studies on *Leptospira interrogans* serovar Lai for the identification and characterization of an iron-regulated protein(s).
    - *In silico* analysis of the genome
    - Experimental approach

II. Role of iron on the expression of sphingomyelinases in *Leptospira* spp.

- *In Silico* analysis of the leptospiral hemolysins, specifically sphingomyelinases.
- Analyse the role of iron on the expression of sphingomyelinases
- Analyse the clinical significance of these virulence determinants

Materials & Methods

3. 1. Chemicals and reagents

Agarose, Bovine Serum Albumin, EDTA, EDDA, 2, 2'-dipyridyl, ethidium bromide, acrylamide, sodium acetate, DMSO, hemin-agarose beads and BCA protein assay kit were obtained from Sigma Aldrich Pvt. Ltd, USA. EMJH base, EMJH enrichment media and Noble agar were purchased from Difco, BD Sciences, USA. pET vector system and Bugbuster Ni-NTA His-bind purification kit was purchased from Novagen, USA. QIA-prep plasmid preparation kit was purchased from QIAGEN GmbH, Germany and DNA or gel band purification kit was purchased from Amersham Biosciences, UK. The DNA labeling kit was obtained from New England Biolabs, INC. USA. Hybond N+ for nucleic acid transfer and DYEnamic ET terminator kit for automated DNA sequencing were from Amersham Pharmacia Biotech, Sweden. X-ray films and intensifying screens were purchased from Kodak, USA. DNA restriction and modifying enzymes, DNA and protein molecular weight markers and RNaseA were purchased from MBI Fermentas, Lithuvania. Nitrocellulose membrane was purchased from Sartorius GmbH, Gottingen. Oligo nucleotide primers of HPLC grade purity were synthesized from Integrated DNA Technology Incorporation, USA. BCIP–NBT and all conjugates were obtained from Bangalore Genei Pvt. Ltd., India. Bacterial grade (*E. coli*) media components and other analytical grade chemicals and solvents were procured from international (Sigma Aldrich / MBI Fermentas) / or local companies (SRL / Himedia / Qualigens / Bangalore Genei Pvt. Ltd).

3. 2. Bacterial strains and plasmids

Pathogenic and non-pathogenic serovars of *Leptospira* spp. and *E. coli* strains and plasmid used in the present study are described in Table 3.

3. 3. 1. Media and growth conditions

Leptospiral working cultures were regularly grown in liquid media (EMJH base supplemented with 10% enrichment media (Difco) at 30°C. The stock cultures were maintained in semi-solid media (0.15% noble agar) at 30°C in screw capped test tubes. Growth was monitored periodically by dark field microscopy.

For cloning experiments, *E. coli* strains were routinely grown in Luria-Bertani (LB) medium at 37°C. Ampicillin (50 μg / ml)/ kanamycin (50 μg / ml)/ chloramphenicol (34 μg / ml) was added to the medium as required for the appropriate strains (as indicated in Table 3).

3. 3. 2. Establishment of low iron and high iron conditions for leptospiral growth

Leptospiral strains were initially grown in normal liquid media at 30°C and after attaining mid log phase growth (reaches within 10 days), the cultures to be made iron deficient were added with iron chelators like 150 μM 2, 2'-dipyridyl / 200 μM EDDA was added gradually over a period of two days. As leptospires are highly sensitive to low iron concentrations and iron chelators, step-wise lowering of iron was done. These cells were centrifuged at 10, 000 rpm for 20 min and then re-suspended overnight in iron-free medium (2% BSA) that was pre-incubated overnight with 150 μM 2, 2' -dipyridyl / 200 μM EDDA. An identical set of culture without chelator representing iron-replete organisms was maintained. All the cultures were harvested

and analyzed for the expression of iron-regulated membrane proteins (IRMPs). The glasswares were made iron free before use.

3. 3. 3. Chrome azurol S (CAS) agar plates

The siderophore expression was assayed in the culture filtrates of *L. biflexa* by the Universal CAS assay. The blue CAS agar plates were prepared by the method of Schwyn & Neilands (1987) with some modifications. 60.5 mg CAS (Fluka) was dissolved in 50 ml water and mixed with 10 ml of 1 mM ferric chloride solution (1 mM $FeCl_3$.6H2O in 10 mM HCl). This solution was slowly added to 72.9 mg HDTMA (Sigma) dissolved in 40 ml of water. The resultant dark blue liquid was autoclaved. Simultaneously, EMJH medium containing 2% Difco Bacto agar was autoclaved. After cooling the solutions to about 50°C, one volume of the CAS solution was added to 9 volumes of the molten agar, swirled to mix without foaming and immediately poured into sterile petri plates to half-fill the plates. After the agar solidified, another layer of top agar containing only the agar solution (minus CAS) was poured over it. Thus, two layers of the agar can be seen, the lower containing the CAS and the top layer without it. It is necessary to maintain a thin layer of the top agar to appreciate the colour change from blue to orange. In order to achieve growth under high and low iron conditions, iron was added at 4 μg Fe / ml (for high iron growth) and 0.02 μg Fe / ml (for low iron growth) respectively to the molten top agar before pouring. The plates were inoculated with the bacteria from the corresponding liquid culture that was maintained as high iron and low iron conditions. The plates were incubated for 5 days and then photographed.

Table 3- Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Leptospira interrogans serovar Lai strain Lai, Pomona strain Pomona, Autumnalis strain Rachmat, Hebdomadis strain Hebdomadis, Australis strain Ballico, Canicola strain HU IV, Sejroe strain Hardjoprajitno.	Pathogenic	National Leptospirosis Reference Centre (ICMR), Port Blair, India.
Bataviae, Djasmin	Pathogenic	DRDE, Gwalior
Leptospira kirschneri serovar Grippotyphosa strain Moskva V	Pathogenic	National Leptospirosis Reference Centre (ICMR), Port Blair, India.
Leptospira santarosai serovar Sarmin strain CZ-390	Pathogenic	National Leptospirosis Reference Centre (ICMR), Port Blair, India.
Leptospira borgpetersenii Serovars Tarassovi strain Perepelitcin, Ballum strain MUS127	Pathogenic	National Leptospirosis Reference Centre (ICMR), Port Blair, India.
Leptospira biflexa serovar Patoc strain Patoc I	Non – pathogenic	National Leptospirosis Reference Centre (ICMR), Port Blair, India.
Leptospira meyeri serovar Ranarum strain ICF	Non – pathogenic	National Leptospirosis Reference Centre (ICMR), Port Blair, India.
E. coli DH5α	Host strain for cloning	Dr. MS Lab (UOH).
BL21 (DE3) pLys (S)	Host strain for expression	Novagen
Plasmids		
TA vector	Cloning vector: Ap^r	Stratagene
pET 28a (+)	Expression vector: Km^r	Novagen

Ap^r, ampicllin resistant; Km^r, kanamycin resistant.

3. 4. Identification of novel iron-regulated proteins

3. 4. 1. Preparation of whole cell proteins

Leptospira culture was harvested at 10, 000 rpm for 20 min and washed thrice with 10 mM Tris HCl (pH 8.0). The pellet was resuspended in 150 µl of buffer and sonicated for about 5 min (20 sec pulse). 1% SDS was added to the sonicated sample and incubated at 37°C for overnight. The next day, samples were centrifuged at 10, 000 rpm for 10 min to remove the debris and protein was estimated by BCA method.

3. 4. 2. Preparation of outer membrane proteins using Triton X-114 detergent extraction method

Triton X-114 extraction method was followed for outer membrane protein separation (Haake *et al*., 2000). The bacterial cells were harvested by centrifugation at 10, 000 rpm for 20 min and washed thrice with 50 mM Tris HCl (pH 8.0) + 5 mM $MgCl_2$ and then incubated by vortexing overnight at 4°C in 2% Triton X-114 in 10 mM Tris (pH 8.0) + 1 mM EDTA + 150 mM NaCl. The insoluble material (cytoplasmic cylinder) was separated 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 at 2000 x g for 15 min. The bottom detergent phase (outer membrane fraction) was washed thrice with 1 ml of 150 mM NaCl + 10 mM Tris HCl (pH 8.0) + 1 mM EDTA and top aqueous phase (periplasmic proteins) was washed thrice with 2% Triton X-114. The proteins in the detergent phase were precipitated with acetone, solubilized in 10 mM Tris-HCl

buffer with 0.6% SDS, protein estimated by BCA method and subjected to SDS-PAGE.

3. 4. 3. Precipitation of proteins

Acetone precipitation: 9 volumes of chilled acetone was added to the protein sample and incubated overnight at –20°C. Sample was centrifuged at 12, 000 rpm for 20 min and the protein pellet obtained was as such stored at –20°C until further use.

3. 4. 4. Protein estimation

Protein concentration was estimated using BCA protein assay reagent kit (Sigma, USA), following the manufacturer's protocol.

3. 4. 5. SDS-Polyacrylamide gel electrophoresis

Proteins were analysed on SDS-PAGE (Laemmli, 1970). About 20 μg of crude protein or 10 μg of partially purified protein was loaded on mini gels. The separation and stacking gel composition is as follows:

Sample preparation

To the protein sample equal volumes of 2 X sample buffer (0.5 M Tris HCl, pH 6.8, 2% β-mercaptoethanol, 4% SDS, 0.001% bromophenol blue, 20% glycerol) was added, mixed well and boiled for 5 min in boiling water bath. The samples were centrifuged briefly at high speed to remove the debris and the clear supernatant was loaded on to the gel.

Staining and destaining

Electrophoresis was carried out at 100 V after which the gels were stained with

Coomassie brilliant blue R-250 (Sigma). Gels were destained with the destaining solution containing 10% methanol and 10% glacial acetic acid.

Resolving gel preparation

Reagents	Volume (ml) used for casting the gel		
	8%	10%	12%
H_2O	4.6	4.0	3.3
30% acrylamide / bis acrylamide (30:0.8)	2.7	3.3	4.0
1.5 M Tris (pH 8.8)	2.5	2.5	2.5
10% SDS	0.1	0.1	0.1
10% APS	0.1	0.1	0.1
TEMED	0.006	0.005	0.005
Total	10	10	10

5% Stacking gel preparation

Reagents	Volume (ml)
H_2O	3.4
30% acrylamide / bis acrylamide (30:0.8)	0.83
0.5M Tris (pH 6.8)	1.25
10% SDS	0.05
10% APS	0.05
TEMED	0.005
Total	5

3. 4. 6. Western blot analysis

The SDS-PAGE gel was electrophoretically blotted onto nitrocellulose filter (Towbin *et al*., 1979) with the electro-blotting buffer (48 mM Tris base pH 8.3, 39 mM glycine and 20% methanol) using Broviga transfer apparatus. After the transfer, the proteins were visualized by Ponceau-S (Sigma) staining solution. The membrane was then blocked for 1 h with 5% non fat milk (NFM) solution dissolved in Tris-buffered saline (TBS-150 mM NaCl and 50 mM Tris.Cl pH 7.5) containing 0.01% Tween-20. The blot was then washed thrice with TBS buffer containing 0.01% Tween 20 for 10 minutes. After the third wash, the membrane was incubated with TBS buffer containing 1% NFM and anti-LhbpA / anti-Sph_{638} antibodies / clinical serum samples at 1:250 dilution for overnight at 4°C. The blot was washed; added alkaline phosphatase (ALP) conjugate (secondary antibody) at a dilution of 1:500 and the membrane was incubated for 1 h at RT. The blot was developed by addition of BCIP and NBT substrate.

3. 5. *In-silico* analysis

Nucleotide sequences were analyzed using standard bioinformatics tools. BLASTN and BLASTX were used to search NCBI database (and LeptoList) for *Leptospira* genome sequences. CLUSTALW (Thompson *et al*., 1994) for multiple sequence alignment and the NCBI Conserved Domain Database for identifying conserved domains on protein sequences.

Sequence analysis and construction of phylogenetic tree for proteins was done using ClustalX (Thompson *et al*., 1997) and MEGA 3.1 (Kumar *et al*., 2004) programmes. Bacterial promoter analysis was done using BPROM programme.

3. 5. 1. Homology search and 3D structure prediction

Using ferric enterobactin receptor FepA of *E. coli* (P05825), a TBLASTN search was done against the genome of *L. interrogans* serovar Lai to identify the homologous region. The homologous sequence (Locus tag LB191) from the leptospiral genome was taken along with 3000 bases upstream for identifying regulatory elements. The gene region was translated and was subjected to sequence alignment with FepA of *E. coli* 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 coordinates of the 2.40 A^{o} resolution structure of *E. coli* FepA were taken from Protein Data Bank.

3. 5. 2. Model building of LhbpA

The 3D structure of *E. coli* FepA protein (1FEP, RCSB PDB) was chosen as template for model building of the reference protein LhbpA (Leptospiral hemin-binding protein A, earlier mentioned as LEP_IRMP in the Sritharan *et al*., 2005 manuscript) using Insight II Modeler (Version 2000, Accelrys Inc.). The method (Sali & Blundell 1993, Sali *et al*., 1995) 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 were 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.

3. 6. Cloning and expression studies

3. 6. 1. Genomic DNA Isolation

Genomic DNA was isolated by using the standard detergent-Proteinase K lysis method (Marmur, 1961) as modified by J. L. Johnson (Virginia Tech, Blacksburg, VA). Two loopful of leptospiral cells were suspended into 400 µl of TE buffer (pH 8.0) and was treated with 50 µl lysozyme (stock solution 10 mg / ml) for 1 h at 37°C. The clear solution was then treated with 6 µl Proteinase K (stock solution 10 mg / ml) and 70 µl SDS (10%) and the mixture was incubated in a water bath at 60°C for 10 min with mild shaking. The sample was brought to room temperature and to this an equal volume of phenol: chloroform (1:1) was added and further incubated for 20 min at room temperature. The sample was centrifuged at 12, 000 g for 10 min at room temperature. The top layer was separated (avoid interface) and mixed with 1 / 100 volume of RNase A stock solution (10 mg / ml), and incubated at 37°C for 30 min. The above phenol: chloroform step was repeated again. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute alcohol at –20°C overnight and centrifuged at 12, 000 rpm for 15 min. The DNA pellet obtained was washed with 70% aqueous ethanol. The DNA pellet was air dried overnight and dissolved in an appropriate volume of sterile TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at 4°C. The concentration of DNA was determined

spectrophotometrically and the quality of DNA was checked by agarose gel electrophoresis.

3. 6. 2. DNA amplification by PCR

The 2148 bp Lb191 (*lhbpA* gene) and 638 bp nucleotide sequence encoding the common region of sphingomyelinase C precursor (*sph*1, *sph*2, *sph*3) and pore forming hemolysin (*sph*H) were amplified using forward primers with *NdeI* restriction site and reverse primers with *HindIII* restriction site (synthesized from Integrated DNA Technology Incorporation, USA) (Table. 2). PCR amplification was carried out in a PTC-200 thermal cycler from MJ research. The template included DNA purified from *L. interrogans* serovar Lai. 20 ng of the genomic DNA was added to the 50 µl PCR mix containing 5 µl of 10 X PCR buffer, 0.16 µg each of forward and reverse primers, 1 µl of dNTP mix (10 mM each) and 2.5 units of *Pfu* DNA Polymerase / *Taq* DNA Polymerase. An initial denaturation step (5 min, 95°C) was followed by 30 cycles of amplification (1 min at 95°C, 1 min at 50°C and 2.3 min at 72°C) and (1 min at 95°C, 1 min at 55°C and 1 min at 72°C) for *lhbpA* and *sph* respectively. The terminal delay was set at 72°C for 10 min for directional cloning and for 30 min for TA cloning. The programs ended at 4°C for cooling. Products were electrophoresed in 1.0% agarose gel and visualized under UV light by ethidium bromide staining.

3. 6. 3. Cloning strategies

PCR, RT-PCR, Southern blot, cloning and expression studies were done following the standard molecular biology protocols (Sambrook *et al*., 1989)

Table 4-Primers used in the study

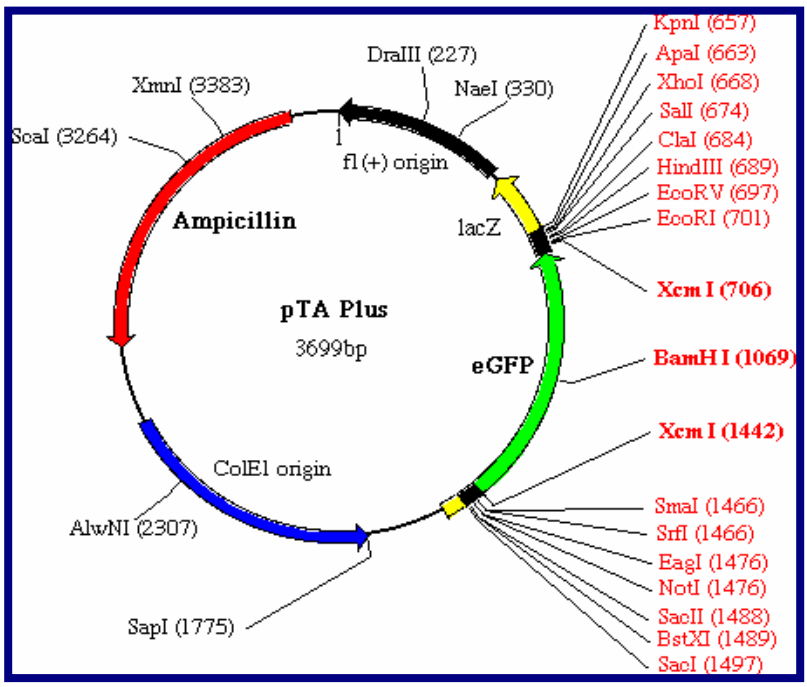
S.No	Gene	Primers
1.	*lhbpA*	**L 1**- (5'-GGG AAT TCC ATA TGT CAT CCA ACC ATT CGA TG -3') **L 2**- (5'-CCC AAG CTT TTA AAA GTG GGC CGA GAA TC -3')
2.	*lhbpA* -1449	**L 3**- (5'-GGG AAT TCC ATA TG G AAT TCA ATA CCA CAG CCA ACA TGG G-3') **L 2**- (5'-CCC AAG CTT TTA AAA GTG GGC CGA GAA TC-3')
3.	Sph_{638}	**S 1**- (5'-GGG AAT TCC ATATG ATG GGA GGA GTT GTC ATT-3') **S 2**- (5'-CCC AAG CTT TTA CCT GAC TTT GTA GGA GT-3')

3. 6. 3. 1. Construction of the bacterial expression vector

The full-length 2148 bp PCR amplified product with T overhangs (last extention step done for 30 min at 72°C) was ligated into TA cloning vector (Stratagene) and the recombinant plasmid was transformed into *E. coli* DH5 α cells. The plasmid was isolated and the 2148 bp insert was digested with *NdeI* and *HindIII* restriction enzymes and then ligated into *NdeI* and *HindIII* sites of T7 RNA polymerase expression vector pET 28a (+). The ligated samples were transformed into *E. coli* host DH5α. The transformants harbouring the respective inserts were screened by PCR and were used for plasmid preparations. The plasmid was then transformed into *E. coli* BL21 (DE3) pLys (S) and used for expression studies. The restriction map of the recombinant plasmid and their orientation were confirmed by restriction analysis using suitable restriction endonucleases.

The PCR amplified and restricted digested 638 bp *sph* fragment was cloned directly into the suitable sites of pET 28a (+) vector.

(a)



(b)

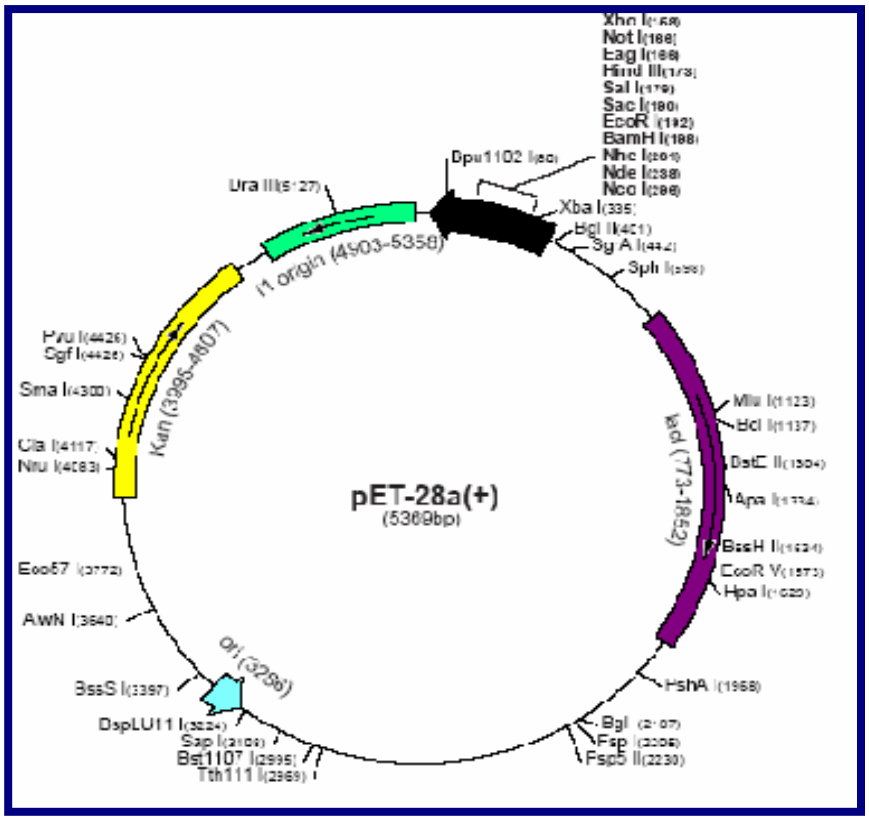


Fig. 10 - Vectors. Panel (a) represents TA cloning vector (Stratagene) and panel (b) represents pET 28a (+) expression vector (Novagen)

3. 6. 3. 2. Restriction digestion

Restriction digestion of the plasmid DNA and the insert was carried out in a total volume of 20 µl containing 0.5 µg of plasmid DNA, 2 µl of appropriate 10 X restriction enzyme buffer, 1 µl BSA (1 µg / µl, if necessary), 15 µl of double distilled water and 1 Unit of restriction enzyme. The reaction was incubated at 37°C for 1 h and the digestion pattern was analyzed on 1% agarose gel.

3. 6. 3. 3. Ligation of DNA fragments

The following principle was used to calculate the concentration of fragment and plasmid DNA needed (3:1 ratio of fragment to vector) for ligation reaction (Sambrook & Russel, 2001). Fragment size / vector size X 100 X 3 where "100" denotes the amount of vector DNA (ng) and "3" denotes the number of times of fragment DNA (ng) required for ligation. The ligation reaction was carried out in a total reaction volume of 20 µl containing 100 ng of restriction digested vector DNA, appropriate amount of fragment DNA, 2 µl of 10 X ligase buffer, 1 µl of T4 DNA ligase (5 U / µl) and sterile double distilled water. The reaction was incubated at 16°C overnight. After completion of the reaction, the sample was used for transformation.

3. 6. 3. 4. Bacterial transformation

The *E. coli* strains containing the recombinant plasmids were grown at 37°C either on solid medium (1.5% agar) or in the liquid LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract). Liquid cultures were grown initially in 2 ml of LB medium in a test tube for plasmid isolation.

a) Preparation of *E. coli* competent cells: 1 ml of *E. coli* DH5α cells from an overnight grown culture was inoculated in 100 ml of LB medium without antibiotic. The cells were grown till they reached a cell density with an A_{600} of 0.6. Cells were harvested into pre-cooled 50 ml Falcon tubes by centrifugation at 3000 rpm for 10 min at 4°C. All the operations were performed under sterile conditions at 4°C. The cells were then re-suspended into 15 ml of 0.1 M $CaCl_2$ and incubated in ice for 30 min. This suspension was centrifuged at 3000 rpm for 10 min. The resultant pellet was resuspended into 1 ml of 0.1 M $CaCl_2$ (in 10% glycerol), dispensed into 200 µl aliquots, frozen and stored at -80°C for future use.

b) Transformation of the competent cells: The competent cells were thawed on ice and added 1-5 ng of plasmid DNA or 100 ng of ligation mix. The suspension was carefully mixed with pipette tip and incubated in ice for 30 min. The sample was subjected to heat shock at 42°C for 90 sec and then incubated in ice for 2 min. 800 µl of liquid LB medium was added and the bacterial suspension was incubated at 37°C with shaking for 1 h. Aliquots of the suspension were spread evenly on LB agar plates supplemented with an appropriate antibiotic. The plates were incubated at 37°C overnight. Single colonies were picked up and tested for the presence of insert by plasmid mini preparation and colony PCR.

3. 6. 3. 5. Minipreparation of plasmid DNA

A single colony of the *E. coli* strain DH5α, obtained as described above, was inoculated into 5 ml of LB medium containing the appropriate antibiotic and incubated overnight with shaking at 37°C. An aliquot of 1.5 ml of the culture was transferred to

a 1.7 ml tube and centrifuged for 5 min at 12,000 rpm at 4°C. The supernatant was removed by aspiration. The pellet was suspended in 100 µl of solution-I (50 mM glucose, 25 mM Tris.Cl pH 8.0, 10 mM EDTA pH 8.0) by vortexing. Then 200 µl of freshly prepared lysis solution (0.2 N NaOH, 1% SDS) was added and the contents were mixed and stored at room temperature for 5 min. Then the solution was neutralized by 150 µl of 3 M potassium acetate pH 5.2, mixed by inversion and stored on ice for 10 min. The cellular debris was removed by centrifugation at 14,000 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 phenol:chloroform (1:1). The plasmid DNA in the aqueous phase was precipitated with 0.6 volume of isopropanol. The DNA pellet was washed with 70% ethanol, air-dried, dissolved in TE and stored at –20°C. High quality plasmid DNA for sequencing purpose was isolated using QIA-prep spin mini-prep kit according to manufacturer's instructions.

3. 6. 3. 6. Analysis of clones by colony PCR

About 50-60 transformed colonies were picked, streaked on selection medium 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.

3. 6. 3. 7. Confirmation of cloned genes by sequencing

DNA sequencing was carried out on a MegaBace 500 automated DNA sequencing system by the dideoxy termination method (Sanger *et al*., 1977) using

DYEnamic ET dye terminator cycle sequencing kit for MegaBace. (This was done in the laboratory of Prof. A. R. Reddy, University of Hyderabad).

3. 6. 4. Expression of recombinant LhbpA and Sph_{638} proteins in *E. coli*

E. coli strain BL21 (DE3) pLys (S) (Novagen) containing the expression vector carrying the recombinant *lhbpA*-ORF and *sph*-ORF was grown in LB medium at 37°C in an orbital shaker until the A_{600} of the culture reached 0.6-0.8. Then the cells were induced with 1 mM IPTG (Sigma) and allowed to grow for an additional three hours. Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C and suspended in Bug Buster reagent (Novagen) (5 ml per gram of wet cell paste). The insoluble cellular debris was removed by centrifugation at 16,000 rpm for 20 min at 4°C. The supernatant was analysed on 8% (rLhbpA) or 10% ($rSph_{638}$) SDS-PAGE.

3. 7. Purification of recombinant LhbpA and Sph_{638} protein

Purification of the hexa-histidine tagged rLhbpA from the *E. coli* whole cell lysate was done by using Bug Buster His-bind Ni-NTA resin affinity chromatography (Novagen purification kit). The protocol was followed according to the manufacturer's instructions.

The crude total protein extract from *E. coli* was applied onto a Ni-NTA His-bind resin column that was pre-equilibrated with binding buffer (4 M NaCl, 160 mM Tris.HCl, 40 mM Imidazole, pH 7.9). The column was washed with 10 volumes of binding buffer followed by 6 volumes of wash buffer (4 M NaCl, 160 mM Tris.HCl, 48 mM Imidazole, pH 7.9). The column was further washed with the wash buffer until the A_{280} of the flow-through was less than 0.01. The bound protein was eluted with 6

volumes of elution buffer (2 M NaCl, 80 mM Tris.HCl, 4 M Imidazole, pH 7.9). The fractions of interest were pooled and analysed for rLhbpA on an 8% SDS-PAGE gel.

The expressed $rSph_{638}$ was found to be present in the insoluble fraction after sonication. The insoluble fraction containing the expressed $rSph_{638}$ protein was separated on 5-20% standard SDS-PAGE gradient gel and the $rSph_{638}$ gel slice was excised and eluted into 20 mM Tris buffer pH 8.0 containing 4% SDS. The eluted protein was again resolved on 10% gel by SDS-PAGE and purified $rSph_{638}$ gel slice was excised and was used for immunization of rabbits.

3. 8. Raising antibodies against the recombinant protein in rabbit

The preparation of antiserum against the purified rLhbpA and $rSph_{638}$ was done by injecting subcutaneously 100 µg of the protein (or protein band) emulsified in Freund's complete adjuvant and the subsequent booster injections were given every alternate week after 3 weeks of primary injection using 50 µg of protein with Freund's incomplete adjuvant (http://www.abgent.com/downloads/polyclonal_antibody.pdf.).

3. 9. Studies on rLhbpA: Characterization studies

3. 9. 1. Affinity chromatography using hemin-agarose beads

Hemin-agarose binding was performed essentially as described by Lee (1992) with little modifications. 200 µl of hemin-agarose (Sigma Aldrich Pvt. Ltd) was washed with 100 mM NaCl, 25 mM Tris-HCl (pH 7.4). Washing was performed three times by suspension of the agarose beads in 1 ml of buffer, followed by centrifugation (750 *g*, 5 min). In two separate experiments, induced *E. coli* whole cell extract / 20 µg of purified rLhbpA was incubated with the washed hemin-agarose beads for 1 h at

37°C with gentle mixing, centrifuged (750 g, 5 min) and the supernatant was discarded. The hemin-agarose was washed three times as described above, and the bound proteins were eluted by incubation for 2 min with 2% w/v SDS and 1% v/v β-mercaptoethanol in 500 mM Tris HCl pH 6.8. The samples were boiled at 100°C for 5 min, centrifuged and analyzed on an 8 % SDS-PAGE gel.

3. 9. 2. Assay of heme-dependent peroxidase activity

The rLhbpA (0.5-4 μg) was incubated with hemin (20 μg / 100 μl) in a 96 well micro titre plate at 37°C for 1 h. The wells were washed with 1X PBS three times and added 100 μl of TMBZ / H_2O_2 substrate (Bangalore Genei). The reaction was stopped with 1N H_2SO_4 and the peroxidase activity was read at 450 nm. The amount of hemin bound to rLhbpA was calculated from peroxidase activity of known concentrations of hemin.

3. 9. 3. Spectrofluorimetric analysis of rLhbpA upon addition of hemin

Emission spectra was recorded on a Spex Fluoromax-3 fluorescence spectrometer from Jobin-Yvon (Edison, NJ, USA, website: http://www.jobinyvon.com). Slit widths of 3 and 6 nm were used on the excitation and emission monochromators respectively. The integration time was set at 0.3 s. rLhbpA sample, showing $A_{280\ nm}$ of 0.05 was irradiated with light of wavelength 295 nm to selectively excite tryptophan residues of the protein and the emission spectra was recorded above 300 nm. Then added aliquots of 1.5 mM hemin to the protein sample and fluorescence spectra recorded after each addition. The fluorescence intensities were corrected for volume changes before further analysis of the quenching data. All

measurements were performed at 25°C. All quenching experiments were carried out in duplicate, which showed high reproducibility and the average results are reported.

3. 10. Agglutination of live organisms after addition of anti-LhbpA antibodies

The protocol for Microscopic Agglutination Test (Cole *et al*., 1973) normally used for clinical samples was applied to study the agglutination of the serovar Lai upon addition of anti-LhbpA antibodies. The anti-rLhbpA antibodies at dilutions of 1:100 and 1:50 was added to live organisms grown under high and low-iron conditions. The pre-immune serum from the same animal was used as the antibody control.

3. 11. Southern blot analysis

Around 20 µg of genomic DNA isolated from the serovars of *Leptospira* spp. were subjected to digestion with *HindIII* restriction enzyme at 37°C overnight. The digested samples were separated on a 1% agarose gel by subjecting them to a electrophoretic run for 12 h in a 25 cm X 13 cm gel tank (Broviga) with an applied voltage of 0.5 V / cm.

After electrophoresis, the DNA was depurinated in 250 ml of 250 mM HCl solution for 30 min followed by washing with sterile double distilled water. The depurinated gel was denatured for 15 min in 250 ml of denaturation solution (1 M NaCl and 0.5 M NaOH) on a rocker maintained at 15 cycles per minute. The depurinated gel was thoroughly washed using double distilled water and neutralized for 15 min in 250 ml of neutralization solution [1.5 M NaCl and 0.5 M Tris (pH 7.0)] and the gel was vaccu-blotted on the Nylon+ membrane (Amersham) as per the

manufacturer's instructions. The Nylon+ membrane was placed between the Whatmann papers and baked for 2 h at 80°C.

3. 11. 1. Probe preparation-Random Primer method

To 200 ng of the purified *lhbpA* DNA fragment, 5 µl of random primer (DNA labeling kit from NEB, INC. USA) was added and the DNA was denatured by placing the tube in boiling water bath for 5 min and then immediately transferred to ice. To this denatured DNA, 4 µl each of dATP, dGTP, dTTP, 5 µl of $\propto P^{32}$ dCTP, 5 µl of 10 X reaction buffer and 1 µl of Klenow enzyme (10 U) was added and the total volume was made up to 50 µl using sterile water. The reaction mix was incubated at 37°C for 1 h and the synthesized probe was purified using Sephadex G-50 spin column.

The specific activity of the resultant probe was calculated as follows: cpm/µg = cpm X 2.5 X total volume of the probe X 1000 / 40; where "2.5" is a Czrenkoff factor.

3. 11. 2. Hybridization and development of the blot

The nylon membrane after baking was placed inside a hybridization bottle containing 150 µl / cm^2 prehybridization solution (0.5 M Na_2HPO_4, 7 % SDS and 1 mM EDTA, pH 7.0) and incubated for 30 min in a hybridization oven maintained at 65°C.

The synthesized probe was denatured in a boiling water bath for 5 min and immediately transferred to ice for 10 min. The prehybridization solution was removed, an equal volume of fresh prehybridization solution was added, followed by the addition of the denatured probe and allowed to hybridize overnight at 65°C in the hybridization oven.

The hybridization solution was discarded and the membrane was washed in a solution containing 2 X SSC and 1% SDS, for 30 min at 65°C. The blot was then washed twice with 0.5 X SSC solution containing 1% SDS for 10 min. Finally, the membrane was rinsed in 2 X SSC solution at room temperature, air-dried and covered with Saran wrap for autoradiography.

An X-ray (Kodak) film was placed over the membrane wrapped in Saran in the X-ray cassette and kept at –70°C by wrapping it in a black cloth for suitable length of time (usually overnight). The film was allowed to expose for sufficient length of time depending on the intensity of counts recorded using a GM counter. The autoradiogram was developed by rinsing it in the developer solution for 2 min followed by a brief wash using distilled water for 1 min. Finally the film was immersed in fixer solution for 2 min and washed thoroughly using tap water and air-dried.

3. 12. Studies on $rSph_{638}$

3. 12. 1. Dot-blot analysis with $rSph_{638}$

Clinical serum samples from patients with leptospirosis, tested positive by MAT, were used to show immunoreactivity against $rSph_{638}$ by dot blot test. Around 2 µl containing 4 µg of $rSph_{638}$ was spotted on the membrane, serum (1:250) was added and allowed to incubate overnight. Then the anti-human ALP conjugate (1:500 dilution) was added, incubated for 1 h and the blot was developed using the ready-to-use BCIP and NBT substrate (Bangalore Genei). The density of the individual colored spot was measured using densitometer (Bio rad GS 800 densitometer and the software used was Quantity ONE from Bio rad).

3. 12. 2. RT-PCR to demonstrate the iron-regulated expression of LhbpA and Sph_{638}

3. 12. 2. 1. Extraction of total cellular RNA

The bacterial cell pellet was suspended in Trizol buffer (1ml) (Invitrogen) and incubated in ice for 30 min. Chloroform (200 µl) was added to the above mixture and centrifuged at 12, 000 rpm for 15 min. The aqueous upper phase was precipitated with equal volumes of isopropanol, incubated at - 80°C for 20 min and centrifuged at 12, 000 rpm for 15 min at 4°C. The pellet obtained was washed with 70% ethanol and was resuspended in RNase free water. The RNA was treated with DNase and the RNA prepared was checked on a 1.2% denatured agarose formaldehyde gel and the concentration was estimated spectrophotometrically.

3. 12. 2. 2. RT-PCR

Using the RNA as template, RT-PCR was performed using "SUPERSCRIPT™ One-Step RT-PCR" kit / Two-step RT-PCR kit obtained from Invitrogen.

Construction of cDNA (20 µl) was done using 4 µg of the total RNA, 0.16 µg of gene specific reverse primer, 5 X buffer, dNTP mix, 0.1 M DTT and Thermoscript (taken as recommended in the manufacturer's guidelines) and the mixture was incubated at 50°C for 1 h. Sample was inactivated at 75°C for 10 min and column purified before PCR analysis.

PCR amplification was done as per standard protocol with the inclusion of 1 µl DMSO using following conditions: an initial denaturation step (5 min, 95°C) was

followed by 36 cycles of amplification (40 sec at 95°C, 1 min at 50°C and 4 min at 72°C) and (40 sec at 95°C, 1 min at 55°C and 1 min at 72°C) for *lhbpA* and sph_{638} respectively with a final extension step of 15 min at 72°C in a PTC-200 thermal cycler from MJ research. The amplified samples were analysed on a 1% agarose gel.

3. 13. Hemolysis assay

Standard procedure for hemolysis assay by Hoffman group available at http://www.bioeng.washington.edu/home/SOP/H/hemolysisassay.pdf was followed in order to study the hemolytic effect shown by the whole cell sonicate and culture filtrate proteins of *L. borgpetersenii* serovar Ballum grown under high iron and low iron conditions.

3. 13. 1. Sheep Red Blood Cell (RBC) preparation

5 ml of sheep blood was collected in a EDTA (2 mg / ml) containing screw cap bottle. The blood sample was centrifuged at 3000 rpm for 5 min at 4°C in graduated 15 ml falcon tube and total volume of blood was marked on side of the tube. Plasma was removed, the sheep erythrocytes were washed 3 times with freshly prepared 150 mM NaCl and then resuspended in 0.1 M phosphate buffer (pH 7.4). Working solution of RBC suspension was made by diluting 1 to 10 with the phosphate buffer. The standardization of the RBC suspension and phosphate buffer to be used for the assay was done before the experiment by using different concentrations of these solutions in combination.

The whole cells sonicate and culture filtrate proteins (working solution 1 mg / ml) were prepared freshly. To 0.1 M phosphate buffer (pH 7.4) 50 μg of whole cell

sonicate / 1 mg culture filtrate protein, was added in a total volume of 800 µl, followed by addition of 200 µl of the sheep RBC suspension. The samples were mixed by inversion and incubated in water bath at 37°C for 1 h.

200 µl of the sheep RBC suspension was added to a) 800 µl of phosphate buffer (pH 7.4) (isotonic solution) and b) 800 µl of deionized water (hypotonic solution) served as negative and positive control respectively

Sample analysis

Samples were removed from the water bath and centrifuged at 12,000 rpm for 5 min at 4°C. The absorbance of the supernatant was taken at 541 nm with phosphate buffer (pH 7.4) as blank.

The % hemolysis was calculated using the formulae as follows:

$$\% \text{ Hemolysis} = \frac{(\text{Absorbance of sample}) - (\text{Absorbance of blank})}{\text{Highest absorbance for positive control}} \times 100$$

Results

4. PART-I: STUDIES ON IRON-REGULATED PROTEINS

4. 1. Studies on *L. biflexa* serovar Patoc strain Patoc I grown under high iron and low iron conditions

4. 1. 1. Ability of *L. biflexa* serovar Patoc I to remove iron from CAS agar plates.

When plated on CAS agar plates, *L. biflexa* serovar Patoc strain Patoc I was able to remove the dye bound iron. Plates (a) and (b) (Fig. 11) were photographed after 5 days of growth. Plate (a) represents the low iron cells plated on top agar containing 0.02 μg Fe / ml and Plate (b) represents the high iron cells plated on top agar containing 4 μg Fe / ml. Both the plates were initially blue in colour. They began to turn light green to orange and within 2-3 days of growth, cells in the Plate (a) developed a darker orange red colour. The blue colour disappeared in the Plate (b) also within 3-4 days but the cells remained colorless even after 10 days of incubation.

4. 1. 2. Iron-regulated expression of proteins in *L. biflexa* serovar Patoc strain Patoc I

In the presence of the chelators EDDA, EDDHPA, *L. biflexa* serovar Patoc I expressed four proteins (iron-regulated proteins IRPs) of apparent molecular size 82, 64, 60 and 33 kDa whose synthesis was significantly lower in the control cells (Fig. 12; lanes 1, 4 and 7). The 82 kDa protein was seen in the aqueous extract (Fig. 12a; lanes 2, 3), while the other three bands were seen in the aqueous extract (Fig. 12a; lanes 2 & 3) and the detergent extract of the EDDA and EDDHPA treated cells (Fig.

12b; lanes 5 & 6), with little or no expression in the control cells (lanes 1 & 4 respectively).

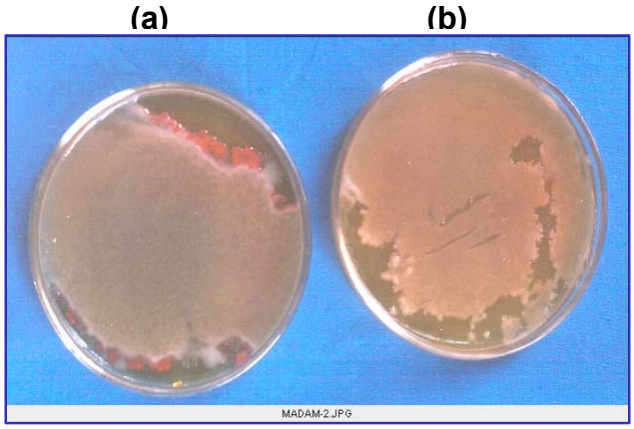


Fig. 11 - Siderophore expression: CAS agar plates. Panel (a): low iron CAS agar plate showing a deep orange color indicating the expression of siderophores. Panel (b): high iron CAS agar plate

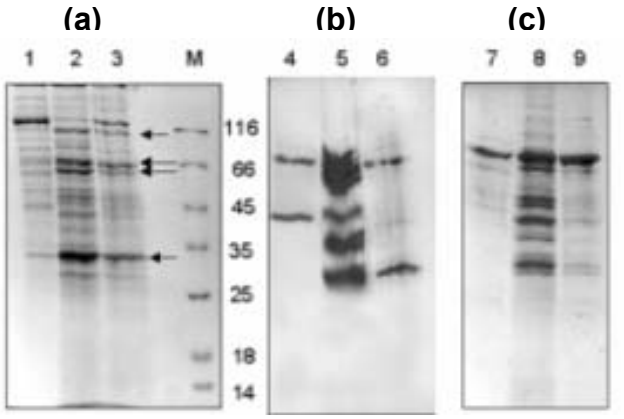


Fig. 12 - SDS-PAGE analysis of the proteins of *L. biflexa* serovar Patoc strain Patoc I grown in the presence of iron chelator. Panel (a): aqueous phase, Panel (b): detergent phase and Panel (c): cell pellet. Lanes 1, 4 and 7: control cells without any chelator, lanes 2, 5 and 8: EDDA treated cells and lanes 3, 6 and 9: EDDHPA treated cells.

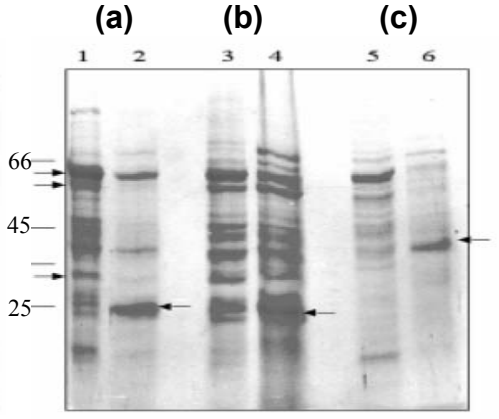


Fig. 13 - SDS-PAGE analysis of the proteins of *L. biflexa* serovar Patoc strain Patoc I grown under high iron (4 µg Fe / ml) and low iron (0.02 µg Fe / ml) conditions. Panel (a): detergent phase, Panel (b): aqueous phase and Panel (c): cell pellet. Lanes 1, 3 and 5 are for cells grown in low iron conditions, lanes 2, 4 and 6 are for cells grown under high iron conditions.

In the alternate protocol in which the cells were grown in medium with 0.02 µg Fe / ml, the 64, 60 and the 33 kDa proteins can be seen in lanes 1 and 3 (Fig. 13), representing the detergent and aqueous phases respectively. These proteins are still seen in lanes 2 and 4, representing the detergent and aqueous extract in cells grown with 4 µg Fe / ml. Also, a prominent 24 kDa band is found in both the detergent and aqueous extracts of cells grown in 4 µg Fe / ml (Fig. 13; lanes 2 and 4), whose synthesis is significantly down-regulated when the iron levels are decreased to 0.02 µg Fe / ml.

4. 2. Iron-regulated proteins in pathogenic *Leptospira interrogans* serovar Pomona strain Pomona

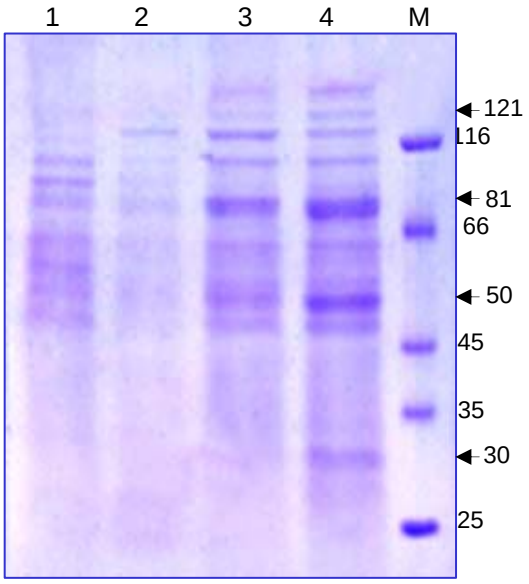


Fig. 14- Iron-regulated proteins in *L. interrogans* serovar Pomona strain Pomona. Lane 1 represent the high iron organism; lanes 2, 3 and 4 represent organism grown in the presence of 100 µM 2, 2' dipyridyl, 200 µM EDDA and 10% serum respectively. Lane M represents the protein molecular weight marker. The whole cell sonicate was used in this experiment. The arrows indicate the iron-regulated protein of molecular mass 121, 81, 50 & 30 kDa

Several iron-regulated proteins (IRPs) were detected in the whole cell sonicate of *L. interrogans* serovar Pomona strain Pomona by SDS-PAGE analysis. In the presence of 2, 2' dipyridyl, there was significant disintegration of the organism and thus low level of proteins were seen in the cell sonicate. In the presence of iron chelator EDDA, an 81 kDa protein was seen. This was also seen in the presence of 10% serum (with 0.02 μg / ml of Fe). In the latter, additional proteins of 121, 50 & 30 kDa was also observed (Fig. 14). Organisms grown under high iron conditions did not express these proteins.

4. 3. Bioinformatic approach: Identification and modeling of a TonB-dependant outer membrane receptor protein from the genome of *L. interrogans* serovar Lai strain Lai

4. 3. 1. BlastP analysis with *E. coli* FepA and identification of LB191, a TonB-dependant outer membrane protein from the genome of serovar Lai.

Using the *E. coli* ferric enterobactin receptor protein FepA as query sequence, (P05825), we identified the homologous sequence (Locus name: LB191 and Swiss Prot - Q8EXL7) from the genome of *L. interrogans* serovar Lai strain Lai by BLASTP analysis (Fig. 15). Leptospiral protein (Q8EXL7) was initially referred to as LEP_IRMP (leptospiral iron-regulated membrane protein) (Sritharan *et al*., 2005) but is henceforth referred to as LhbpA (leptospiral hemin-binding protein A).

The alignment of these two proteins showed that the leptospiral protein showed low similarity (39%) and identity (22%) with the FepA protein (Fig. 16).

Sequences producing significant alignments:		**(bits)**	**Value**
ref|NP_714735.1|	putative TonB-dependent outer membrane rec...	59	9e-10
ref|NP_713329.1|	Hemin receptor [Leptospira interrogans ser...	42	2e-04
ref|NP_710753.1|	conserved hypothetical protein [Leptospira...	41	3e-04
ref|NP_711537.1|	probable TonB-dependent receptor [Leptospi...	35	0.019
ref|NP_712822.1|	probable TonB-dependent receptor [Leptospi...	33	0.043
ref|NP_713685.1|	hypothetical protein [Leptospira interroga...	30	0.47
ref|NP_713648.1|	probable TonB-dependent receptor [Leptospi...	28	1.4
ref|NP_713848.1|	Uracil-DNA glycosylase [Leptospira interro...	28	1.8
ref|NP_710592.1|	Electron transfer flavoprotein alpha-subun...	27	3.1
ref|NP_713438.1|	hypothetical protein [Leptospira interroga...	27	4.0
ref|NP_713155.1|	hypothetical protein [Leptospira interroga...	27	4.0
ref|NP_711900.1|	Cysteine synthase [Leptospira interrogans ...	27	5.2
ref|NP_711229.1|	4-diphosphocytidyl-2C-methyl-D-erythritol ...	27	5.2
ref|NP_713350.1|	Sigma factor sigB regulation protein rsbU ...	26	6.8
ref|NP_710764.1|	Cation efflux system protein CZCA [Leptosp...	26	6.8
ref|NP_714685.1|	similar to putative lipoprotein qlp42 [Lep...	26	6.8
ref|NP_713351.1|	hypothetical protein [Leptospira interroga...	26	6.8
ref|NP_711366.1|	Response regulator receiver domain [Leptos...	26	8.9

Fig. 15 - BLASTP analysis of the genome of *L. interrogans* serovar Lai strain Lai using *E. coli* FepA ferric enterobactin receptor

```
Query: 30  VSHDDTIVVTAAEQNLQAPGVSTITADEIRKNPV----ARDVSKIIRTMPGVNLTGNSTS 85
           V  +  IVVT +    +    ST+  + I +  +    AR+ ++++ T  G+++
Sbjct: 76  VPEESQIVVTGSRGERRLKD-STVATEVISRKKIEASGARNAAEVLETQLGIDVVPFF-- 132

Query: 86  GQRGNNRQIDIRGMGPENTLILIDGKPVSSR--NSVRQGWRGERDTRGDTSWVPPEMIER 143
              G +R + + G+  +  LILIDG+ +S R  N+V            D S    + IER
Sbjct: 133 ---GGSR-VRMLGLDSQYVLILIDGERISGRLNNAV-----------DLSRFKVQNIER 176

Query: 144 IEVLRXXXXXXXXXXXXXVVNIITKKGSGEWHGSWDAYFNAPEHKEEGATKRTNFSLTG 203
           IE+++              V+N+IT++         D   +       G   R NF+  G
Sbjct: 177 IEIVKGASSALYGADAIGGVINLITREA--------DKKLSYEMRTTYGNGSRKNFNTEG 228

Query: 204 PLGDEFSFRLYGNLDKTQADA-WDINQGHQSARAGTYATTLPAGREGVINKDINGVVRWD 262
                +              A ++ N G++     + ATT      G   +D+N  +
Sbjct: 229 EFNTTANMGFRNEYVSGAVSAGYNKNPGYRLV-PNSQATT------GNAYQDLNTGINLT 281

Query: 263 FAPLQSLELEAGYSRQGNLYAGDTQNTNSDSYTRSKYGDETNRLYRQNYALTWNGGWDNG 322
           F P    +      +   LY    QN    + +++ + D  N+ +  ++  T  G  + G
Sbjct: 282 FNPDGKFK-----GKTRILYQHRDQNGVDVTQSKAVF-DRNNKTH--DFLAT--GSLEYG 331

Query: 323 VTTSNWVQYEHTRNSRIPEGLAGGTEGKF-NEKATQDFVDIDLDDVMLHSEVNLPIDFLV 381
               N + +          G     E K+ N +   D +D+   +  L S+  + +D
Sbjct: 332 FGKRNLISFR---------GNISKWENKYYNNQRGSDELDVKQLNSELTSQGTVQLDMEA 382

Query: 382 NQT--LTLGTEWNQQRMKDLSSNTQALTGTNTGGAIDGVSTTDRSPYSKAEIFSLFAENN 439
           ++   +T+G E     ++     ++ +  T          T  RSP  +
Sbjct: 383 SEKHFITVGAESFANELESDRLQSRYVYRTRKAVFFQDEWTVSRSPRIR----------- 431

Query: 440 MELTDSTIVTPGLRFDHHSIVGNNWSPALNISQGLGDDFTLKMGIARAYKAPSLYQTNPN 499
                V PG+R+D  S  GN  +P L     +  +   +     R ++ PS  +
Sbjct: 432 --------VVPGVRYDDDSQFGNQTTPKLAARYDIFQNLVWRASYGRGFRPPSFQE---- 479

Query: 500 YILYSKGQGCYASAGGCYLQGNDDLKAETSINKEIGLEFKRDGWLA-GVTWFRNDYRNKI 558
             LY + +     A G  ++GN +LK E SI     LE+    +L   ++ +RND  N I
Sbjct: 480 --LYLRFEN---PAVGYVVEGNPNLKPERSITINSDLEYSPFSFLTFSLSVYRNDIINLI 534

Query: 559 EAGYVAVGQNAVGTDLYQWDNVPKAVVEGLE 589
           +  Y           +Q  N+ KA   G E
Sbjct: 535 Q--YKFDSNKGREFAEFQLQNIAKAYTRGGE 563
```

Fig. 16 - Basic Local Sequence Alignment of FepA (P05825) and the leptospiral protein Q8EXL7

4. 3. 2. Modeling of LhbpA using Insight II modeller

Using the FepA protein as the reference protein, LhbpA was modelled using the Insight II software. Fig. 17 shows the sequence alignment of the FepA protein and LhbpA, generated by pair-wise alignment algorithm of the homology module of the Insight II program. The 3D structure of LhbpA generated shows the β barrel structure (Fig. 18a) and the plug domain (Fig. 18b). The protein folding of the LhbpA is

identical to FepA (Fig. 19) and the structural alignment of the modeled LhbpA with the crystal structure of the FepA protein (Fig. 20) shows the high degree of structural similarity between them.

Fig. 17 - Alignment of FepA (P05825) and leptospiral protein (Q8EXL7), generated by pair-wise alignment algorithm of homology module in Insight II software. The residues shown in the box are structurally conserved regions (SCRs) and the residues outside the box are structurally variable regions (SVRs)

The RMSD for the backbone atoms of the two structures is 0.7245 A^{o}. The small RMSD can be interpreted to mean that the two structures share common homology and the generated structure is reasonable. The structurally conserved

regions (SCRs) and variable regions (SVRs) in both the structures are displayed in Fig. 17.

4. 3. 2. 1. The β barrel domain

The modeled LhbpA showed the characteristic β barrel structure along with the conserved plug domain and the TonB box. This β barrel domain forms the trans-membrane part of the receptor. It consists of 22 anti-parallel strands, with large loops extending towards the extracellular side and short loops facing the periplasmic side (Fig. 18a).

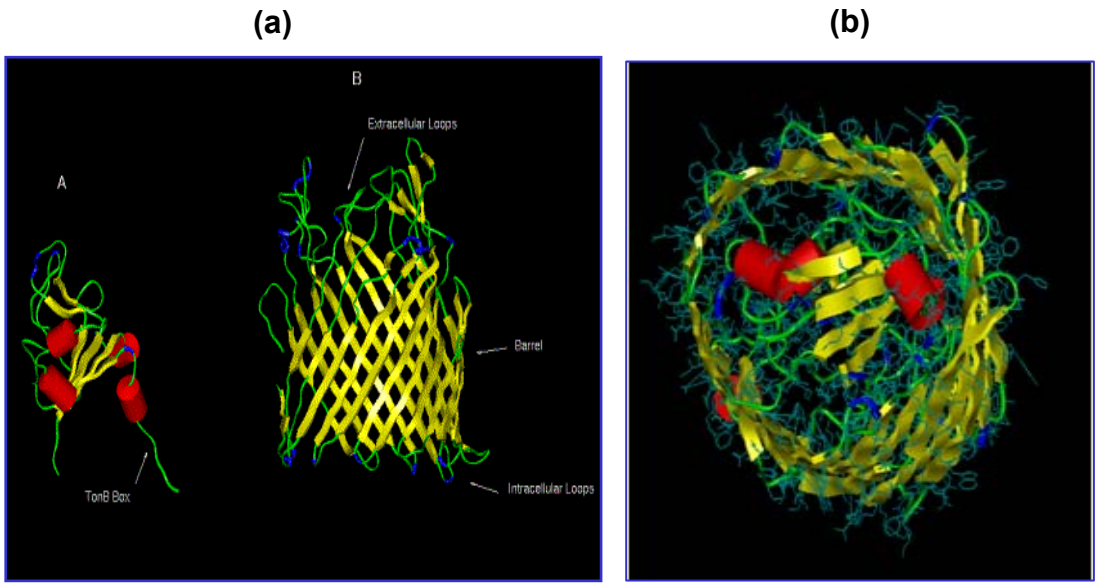


Fig. 18 - Homology modeled 3D structure of LhbpA. The modeled structure showed the 22 anti-parallel strands forming the β-barrel (Fig. 20a-B), interconnected by long extracellular loops and short intracellular loops. The plug domain (Fig. 20a-A) formed by the N-terminal region of the protein consists of six helices and six β strands. The TonB box at the N-terminus can be seen in Fig. 20a-A. Fig. 20b shows the top view of the plug domain of LhbpA.

4. 3. 2. 2. The plug domain

The plug domain is a globular domain at the N-terminal part of the receptor that folds into the β barrel and completely closes it. The plug domain of the LhbpA, consisting of 6 helices and 6 β strands shows a high degree of conservation with the corresponding region of FepA protein of *E. coli*.

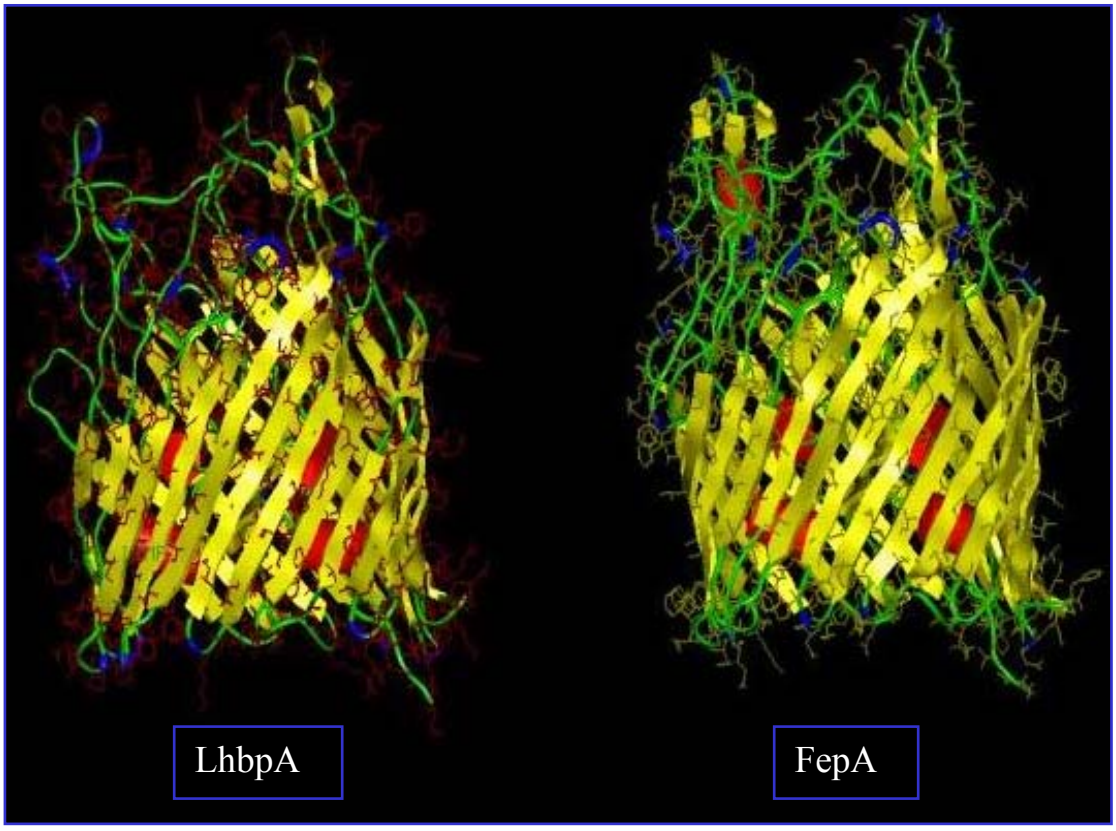


Fig. 19 - 3D structure of the leptospiral LhbpA and FepA of *E. coli*

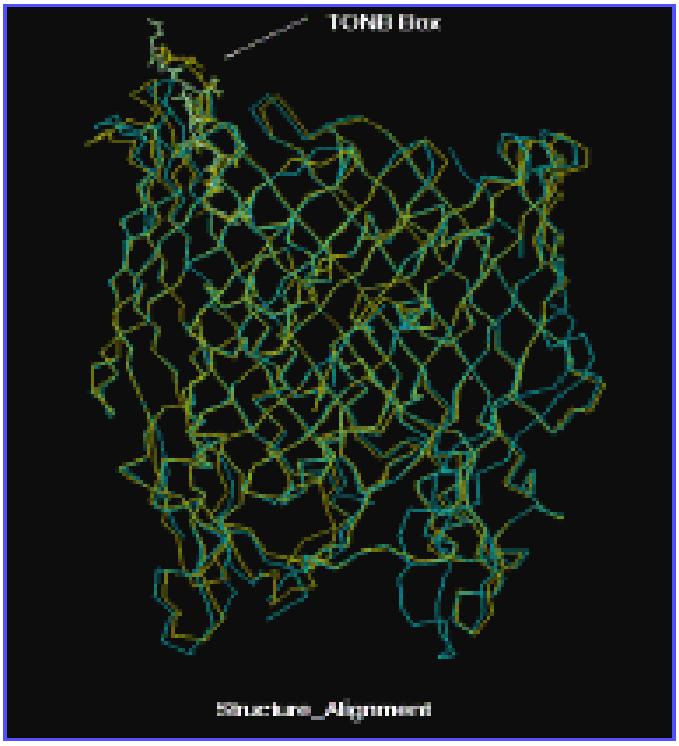


Fig. 20 – Structure alignment. Structural superimposition of the crystal structure of the ferric enterobactin receptor FepA of *E. coli* (blue) on the modeled structure of the LhbpA (yellow)

4. 3. 2. 3. The TonB box

The consensus sequence of the TonB box include

- N-terminal region: (DENF) (ST) (LIVMF) (LIVSTEQ) V X (AGP) (STANEQPK)
- C-terminal region: (LYGSTANEQ) X (3) (GSTAENQ) X (PGE) R X (LIVFYWA) X (LIVMFTA) (STAGNQ) (LIVMFYGTA) X (LIVMFYWGTADQ) X F

respectively.

Based on these consensus sequence patterns, the N-terminal TonB box of LhbpA was identified as **ESQIVVTGS** at 79-87 positions (Fig. 21). All the amino

acids fall in the consensus sequence except the 3rd amino acid Q that is different. The corresponding TonB box in the ferric enterobactin receptor FepA is DDTIVVTAA located at 34-41 positions.

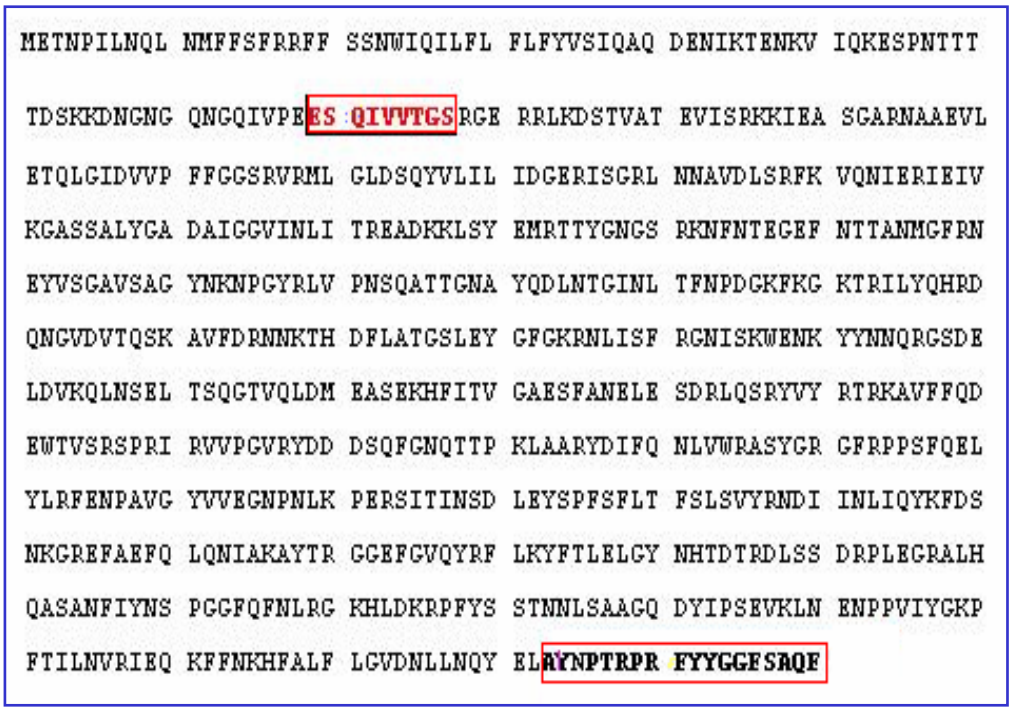


Fig. 21 - TonB box in LhbpA. The TonB box **ESQIVVTGS** at the N-terminus was identified based on the TonB consensus sequence; similarly a conserved sequence at the C-terminus was identified.

To validate our homology modeled LhbpA structure, Ramachandran plot was drawn and the structure was analyzed by PROCHECK, a well-known protein structure checking program. The phi-psi plot is shown in Fig. 22. The % of residues falling in the favourable region is 82.6%, a value that is relatively good.

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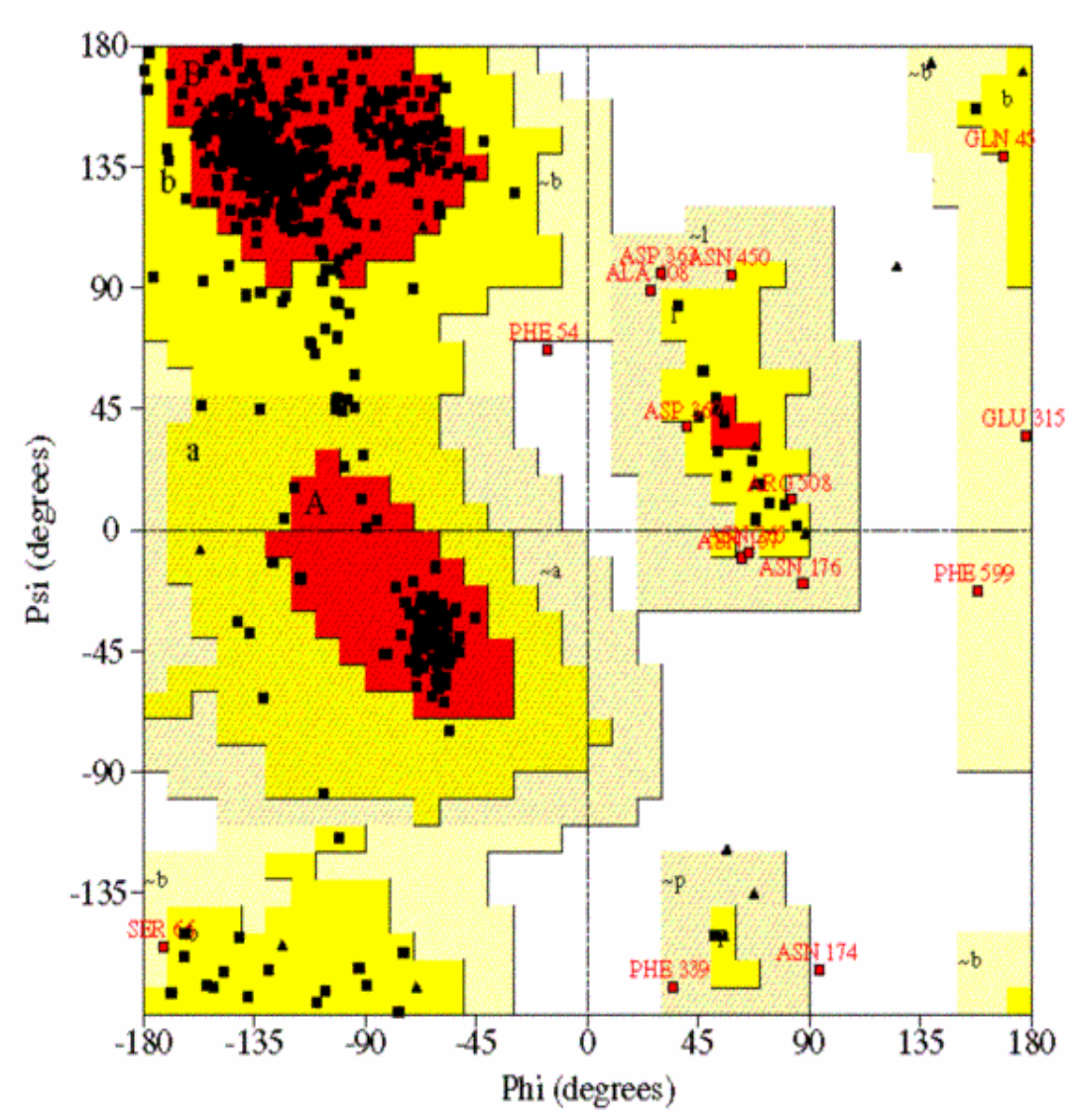


Fig. 22 - Structure validation of LhbpA. Ramachandran plot for the modelled structure of LhbpA generated by PROCHECK software showed 82.6% of the peptide φ–ψ angles falling within the favorable region.

4.4. Homology of LhbpA with other bacterial sequences

LhbpA showed homology with the colicin receptor / vitamin B12 receptor / ferric-siderophore protein receptor / heme-binding protein of several Gram-negative bacteria in the BLASTP search against the Data Bank of microbial genomes (Fig. 23).

Sequences producing significant alignments:		(bits)	Value
gi|2507462|sp|P17315|CIRA_ECOLI	Colicin I receptor precursor	140	9e-33
gi|12644182|sp|P27772|IRGA_VIBCH	Iron-regulated outer membr...	130	1e-29
gi|416728|sp|P06129|BTUB_ECOLI	Vitamin B12 receptor precursor	89	3e-17
gi|20141233|sp|P37409|BTUB_SALTY	Vitamin B12 receptor precu...	79	4e-14
gi|2501236|sp|Q56989|HMUR_YERPE	Hemin receptor precursor	67	1e-10
gi|2507463|sp|P05825|FEPA_ECOLI	Ferrienterobactin receptor ...	65	6e-10
gi|6016198|sp|P31499|HEMR_YEREN	Hemin receptor precursor	57	1e-07
gi|1170593|sp|P14542|IUTA_ECOLI	Ferric aerobactin receptor ...	56	2e-07
gi|6685883|sp|Q9Z3Q5|RHTA_RHIME	Rhizobactin receptor precur...	55	8e-07
gi|19924208|sp|P44795|HGP1_HAEIN	Probable hemoglobin and he...	53	2e-06
gi|18203187|sp|Q9KIV1|HGBB_HAEIN	Hemoglobin and hemoglobin-...	53	2e-06
gi|18202545|sp|Q48153|HHUA_HAEIN	Hemoglobin-haptoglobin bin...	52	3e-06
gi|1175294|sp|P44836|HGP3_HAEIN	Probable hemoglobin and hem...	52	4e-06
gi|18203671|sp|Q9ZA21|HGPA_HAEIN	Hemoglobin and hemoglobin-...	52	4e-06
gi|18203592|sp|Q9X442|HGPC_HAEIN	Hemoglobin and hemoglobin-...	51	9e-06
gi|548479|sp|Q05098|PFEA_PSEAE	Ferric enterobactin receptor...	50	2e-05

Fig. 23 - BLASTP analysis of LhbpA against other microbial genomes

4. 5. PCR amplification and Southern blot analysis of *lhbpA*

PCR amplification using primers L1 & L2 (Table 4) gave the 2148 bp full-length *lhbpA*-2148 bp (Fig. 24). A smaller 1449 bp product, was amplified by heminested PCR using primers L3 & L2.

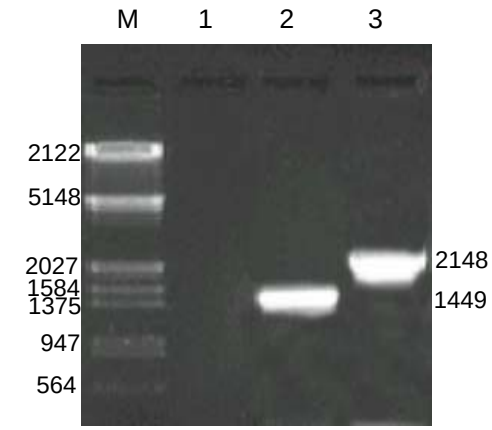


Fig. 24 - PCR amplification of *lhbpA*. Lanes 2 and lane 3 represent the 1449 bp and the full- length 2148 bp products respectively. Lane 1 represents DNA blank and lane M represents DNA marker.

PCR analysis of several serovars was done (Fig. 25) using L1 and L2 primers specific for the full-length 2148 bp product. It is clear that *lhbpA* is absent in the non-pathogenic *L. biflexa* serovar Patoc strain Patoc I (lane 10) and the non-pathogenic *L. meyeri* serovar Ranarum (lane 13). Among the pathogenic leptospires, the 2148 bp full-length *lhbpA* gene was identified in the following serovars - *L. interrogans* serovars Hebdomadis (lane 1), Australis (lane 2), Pomona (lane 3), Canicola (lane 11) and Hardjo (lane 14) and Lai (lane 9). While several smaller PCR products were seen in *L. interrogans* serovar Bataviae (lane 4) and Djasmin (lane 6), *L. borgpetersenii* serovar Tarassovi (lane 5), *L. kirschneri* serovar Grippotyphosa (lane 8), *L. weilii* serovar Sarmin (lane 12), a larger product was seen in *L. interrogans* serovar Autumnalis (lane 7).

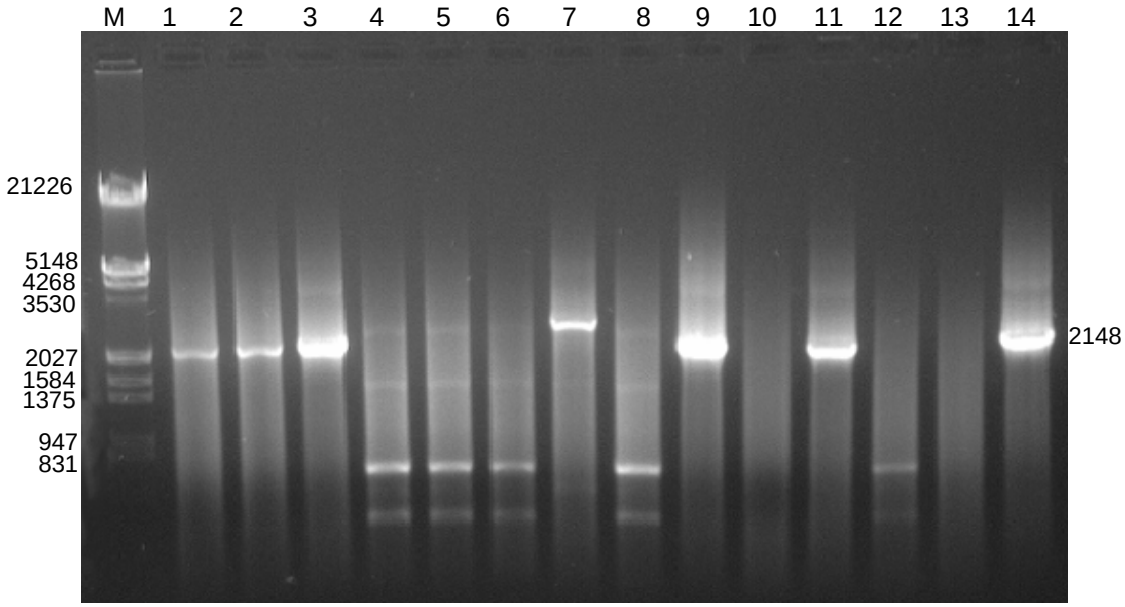


Fig. 25- PCR amplification of full-length *lhbpA* from *Leptospira* spp. The different lanes represent as Lambda DNA / *EcoRI* + *HindIII* double digest molecular weight marker (lane M), *L. interrogans* serovar Hebdomadis strain Hebdomadis (lane 1), *L. interrogans* serovar Australis strain Ballico (lane 2), *L. interrogans* serovar Pomona strain Pomona (lane 3), *L. interrogans* serovar Bataviae (lane 4), *L. borgpetersenenii* serovar Tarassovi strain Perepelitcin (lane 5), *L. interrogans* serovar Djasmin (lane 6), *L. interrogans* serovar Autumnalis strain Rachmat (lane 7), *L. kirschneri* serovar Grippotyphosa strain Moskva V (lane 8), *L. interrogans* serovar Lai strain Lai (lane 9), *L. biflexa* serovar Patoc strain Patoc I (lane 10), *L. interrogans* serovar Canicola strain HU IV (lane 11), *L. interrogans* serovar Sarmin strain CZ-390 (lane 12), *L. meyeri* serovar Ranarum strain ICF (lane 13) and *L. interrogans* serovar Sejroe strain Hardjoprajitno (lane 14).

The serovars were amplified with G1 / G2 primers (Gravekamp *et al*., 1993); Fig. 26 shows the *L. interrogans* species-specific 285 bp product in the respective leptospiral serovars.

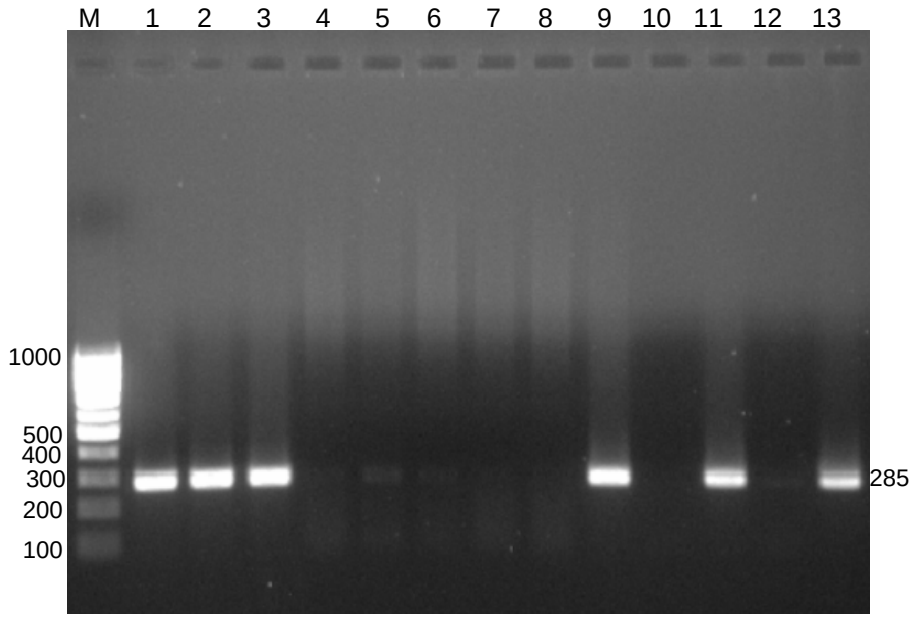


Fig. 26 - G1 / G2 primer-based 285 bp PCR product (Gravekamp *et al*., 1993) specific for serovars of *L. interrogans*. The different lanes represent 100 bp DNA marker (lane M), *L. interrogans* serovar Hebdomadis strain Hebdomadis (lane 1), *L. interrogans* serovar Australis strain Ballico (lane 2), *L. interrogans* serovar Pomona strain Pomona (lane 3), *L. interrogans* serovar Bataviae (lane 4), *L. borgpetersenenii* serovar Tarassovi strain Perepelitcin (lane 5), *L. interrogans* serovar Djasmin (lane 6), *L. interrogans* serovar Autumnalis strain Rachmat (lane 7), *L. kirschneri* serovar Grippotyphosa strain Moskva V (lane 8), *L. interrogans* serovar Lai strain Lai (lane 9), *L. biflexa* serovar Patoc strain Patoc I (lane 10), *L. interrogans* serovar Canicola strain HU IV (lane 11), *L. interrogans* serovar Sarmin strain CZ-390 (lane 12) and *L. interrogans* serovar Sejroe strain Hardjoprajitno (lane 13).

Southern blot analysis of HindIII-digested leptospiral genomic DNA

The genomic DNA probed with the 2148 bp *lhbpA* PCR product is represented in Fig. 27. A single band is seen in *L. interrogans* serovar Lai strain Lai (lane 1), *L. interrogans* serovar Pomona strain Pomona (lane 2) and *L. interrogans* serovar

Autumnalis strain Rachmat (lane 5). No signal was seen in *L. biflexa* serovar Patoc strain Patoc I (lane 3), *L. borgpetersenii* serovar Tarassovi strain Perepelitcin (lane 4) and *L. kirschneri* serovar Grippotyphosa strain Moskva V (lane 6).

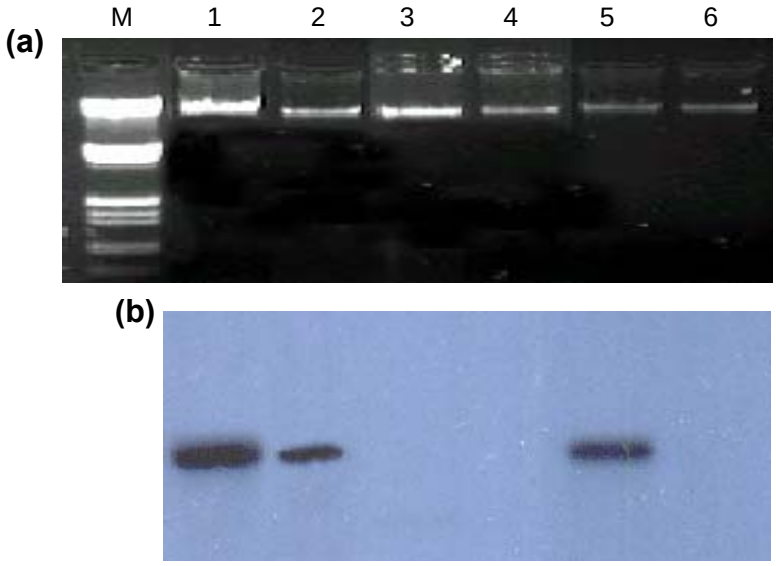


Fig. 27 - Southern blot analysis. Panel (a) shows the respective genomic DNA of *Leptospira* spp., digested with *HindIII*. Panel (b) shows the hybridization signal with *lhbpA* probe. Lanes 1-6 represents *L. interrogans* serovars Lai and Pomona, *L. biflexa* serovar Patoc, *L. borgpetersenii* serovar Tarassovi, *L. interrogans* serovar Autumnalis and *L. kirschneri* serovar Grippotyphosa respectively.

4. 6. Cloning, expression and purification of rLhbpA

The cloning strategy of *lhbpA* is shown in Fig. 28. The full-length 2148 bp *lhbpA* from *L. interrogans* serovar Lai strain Lai, amplified by PCR (Fig. 28a, lane 1) was ligated into TA cloning vector. The *lhbpA* insert from the recombinant TA

plasmid (Fig. 28b) released by double-digestion with *NdeI* and *Hind III* (Fig. 28c, lane 1) was ligated into compatible sites in pET 28a (+) expression vector. The recombinant pET 28a (+) plasmid (Fig. 28d) was transformed into expression host BL21 (DE3) pLys (S). The transformants carrying the gene were screened by colony PCR (Fig. 28e) and restriction analysis. The orientation of the insert was confirmed by *EcoR*V restriction digestion analysis, which gave an expected product size of 2649 bp (Fig. 28f). Before going for expression studies, the positive clone was sequenced (Fig. 29). The recombinant protein, under the control of T7 promoter (Fig. 30a) was expressed by induction with 1 mM IPTG. The colonies of the *E. coli* transformants were small, the growth rate was relatively low and the expression of LhbpA at 37°C was not significant. The expression of the recombinant protein was improved by growing the cells at 22°C (Fig. 30b). The rLhbpA transcript was demonstrated by RT-PCR analysis using whole cell RNA isolated from the LhbpA *E. coli* clones with gene specific L1 & L2 and L3 & L2 primers (Fig. 30c & Fig. 30d). The recombinant protein was purified to apparent homogeneity using Bug Buster Ni-NTA His-bind resin affinity chromatography (Novagen purification kit), as evidenced by the single band of 81 kDa on SDS-PAGE (Fig. 30e).

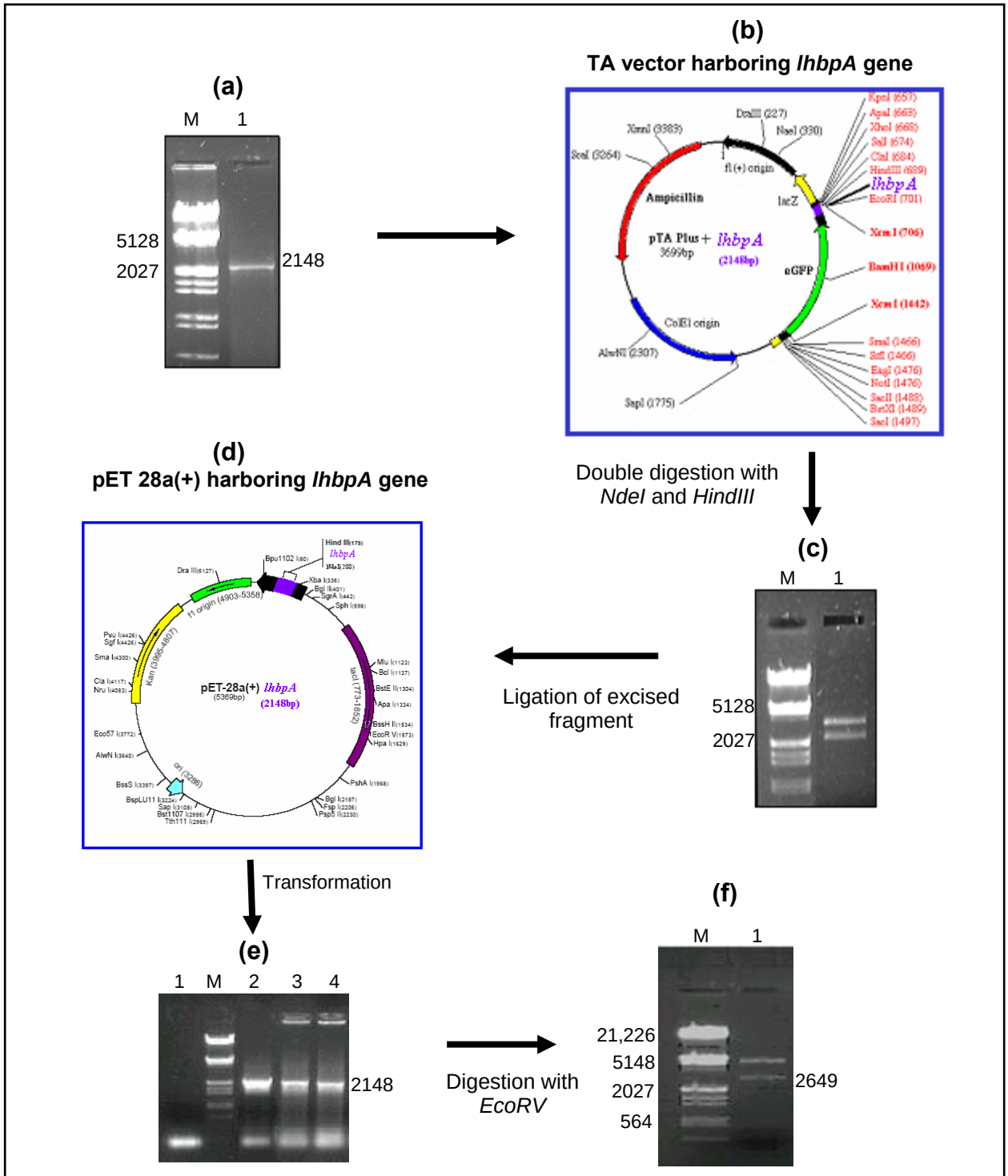


Fig. 28 - Cloning strategy of *lhbpA*. The 2148 bp *lhbpA* amplicon (Panel (a), lane 1) was first cloned into TA vector. The *lhbpA* insert, from the recombinant TA plasmid (Panel b) was restricted digested (Panel (c), lane 1) and ligated into compatible sites of pET 28a (+) expression vector. The recombinant pET 28a (+) plasmid (d) was transformed into expression host BL21 (DE3) pLys (S). The transformants were screened by colony PCR (e).The orientation of the *lhbpA* insert was checked by *EcoRV* enzyme (f). Lane M is the DNA molecular weight marker.

(a)

```
TAATAATAAGCCAGCTTCGTATTGCATTGAGTAATTATCCACTCCCAAAAAAAG
AGCGAAGCTGTTATTGAAAAATTTCTGCTCAATTCTAACGTTGTAAAATCGTAA
ACGGTATTNCCGTAAATCACGGGAGGGATATTCACATTTAATTTAACTTCGCTG
GGAATATAATCCTGTCCTGCCGCTGAAAGATTGTTAGTCGAACTATAAAACGGT
CTTTTGTCTAAATGTTTANCCTCTCAGATTAAATTGGAATCCTCCGGGAGAATT
ATAGATGAAATTATGCAGACGCCTGATGAAGTGCTCTTCCTTCTAAAGGTCTAT
CCGAGCTTAGATCTCTAGTATCCGTATGATTAGTATCACTAATTCCAGGAGTGA
AATATTTTAAGAATCTATATTGAACATCCAAATTCCCCTCCTCTTGTATAAGCC
TTTGCAATGTTCTGTAGTTGAAATTCCGCAAACTCTACTT
```

(b)

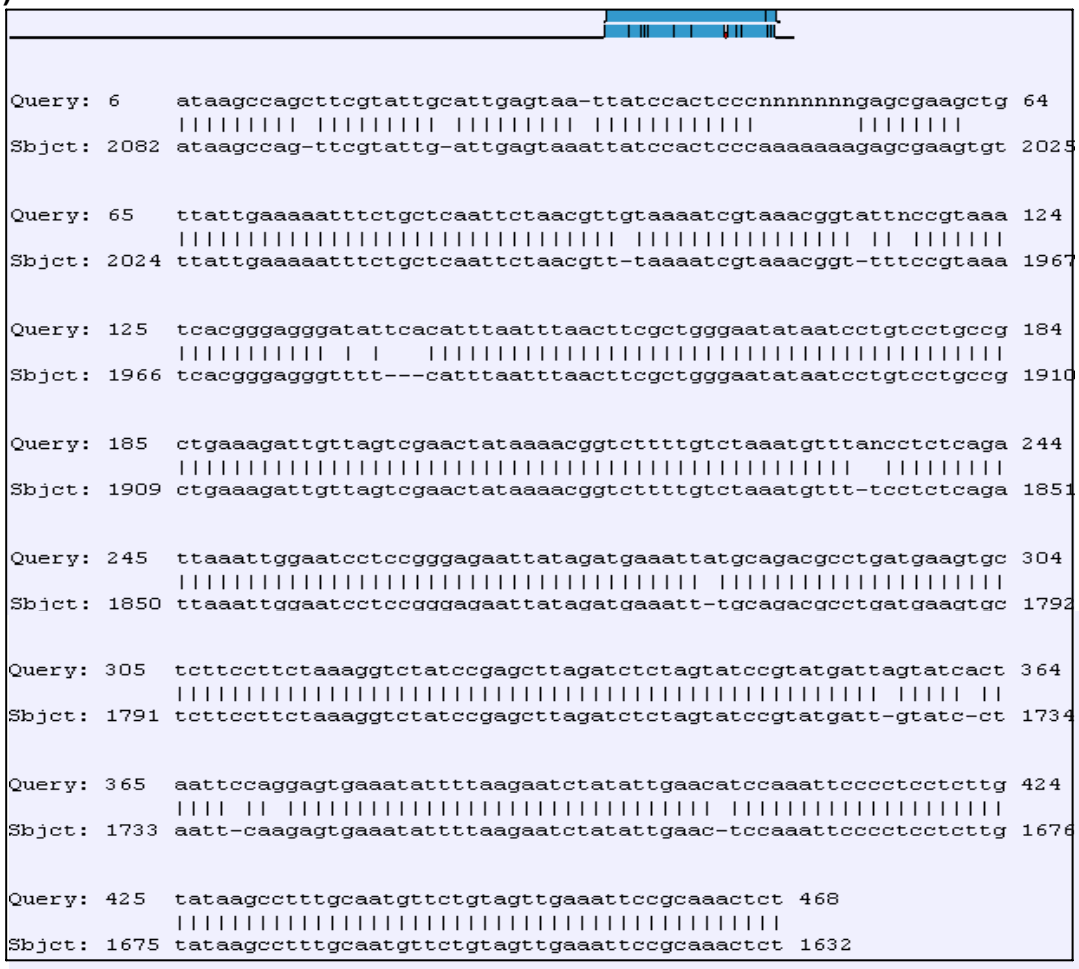


Fig. 29 - Sequence analysis of the *lhbpA* insert. The cloned DNA was sequenced using *lhbpA* gene-specific reverse primer (Panel a); Panel (b) shows the comparison of the insert sequence with the LB191 sequence from genome (similarity is represented as bar diagram).

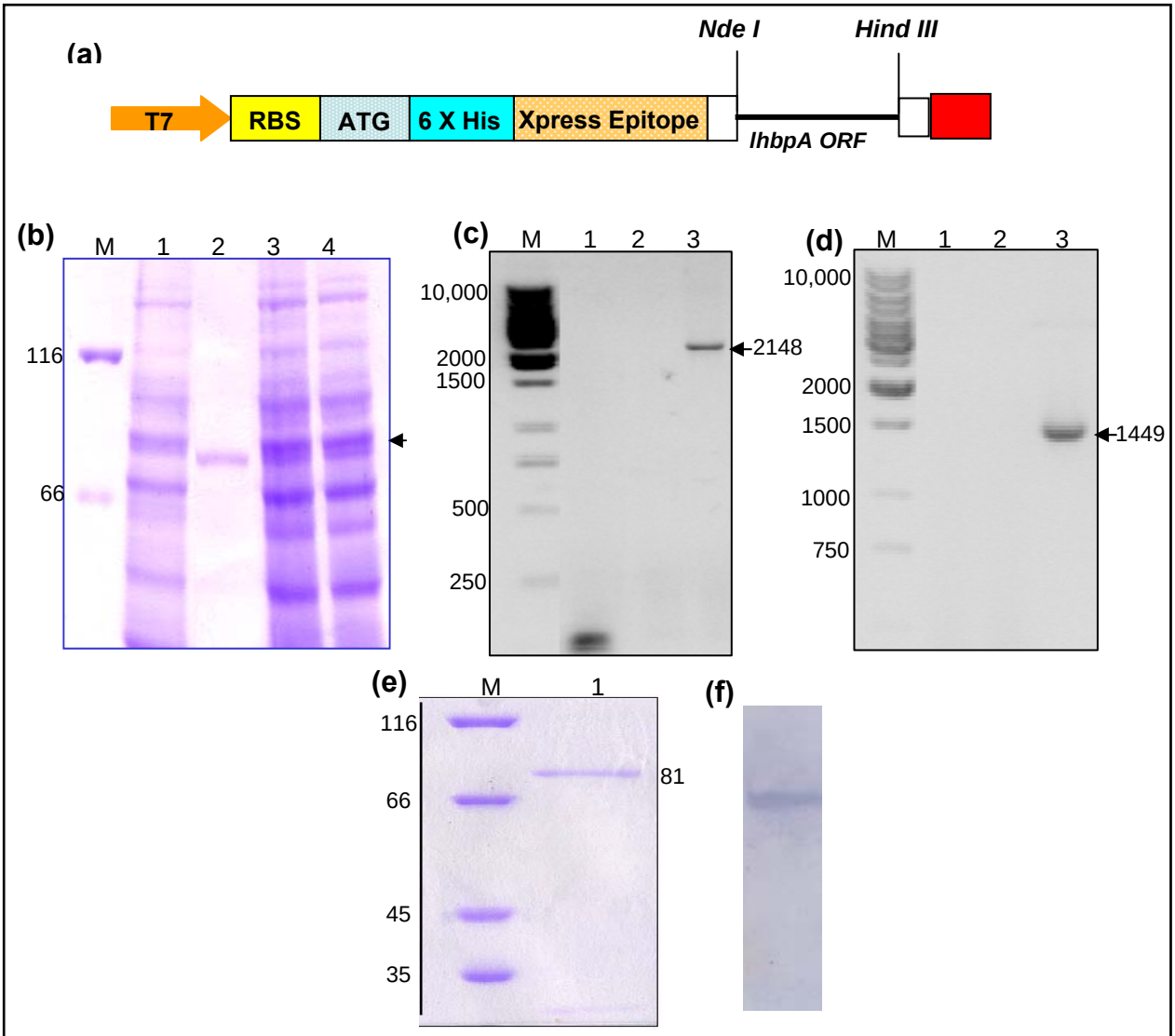


Fig. 30 - Expression and purification of rLhbpA. Panel (a) shows the diagrammatic representation of the expression construct of *lhbpA* in pET 28a (+). Panel (b) shows the SDS-PAGE analysis of the expressed rLhbpA at 2 h (lane 3) and 3 h (lane 4) upon IPTG induction; Lane 1 is un-induced cells. IPTG induced *E. coli* cell sonicate was passed through Ni-NTA His-bind agarose beads for affinity binding and the eluted protein was concentrated using Amicon ultra filtration system. Panel (e) shows the purified rLhbpA as an 81 kDa band (lane 1) and Panel (f) shows the immuno-reactivity of rabbit antibodies raised against rLhbpA. Panel (c) & (d) are the RT-PCR analysis of *E. coli* clones, which shows the expression of rLhbpA (lane 4) and rLhbpA -1449 (lane 4) respectively. Controls included total RNA as template (lane 2) and cDNA of un-induced *E. coli* cells (lane 3). Lane M represents protein / DNA molecular weight marker.

4. 7. Characterization of rLhbpA as a hemin-binding protein

Alignment of LhbpA with other bacterial hemin binding proteins using ClustalX and Mega 3.1 software for tree view (Fig. 31) shows that LhbpA is evolutionarily related to hemin-binding proteins of other bacteria.

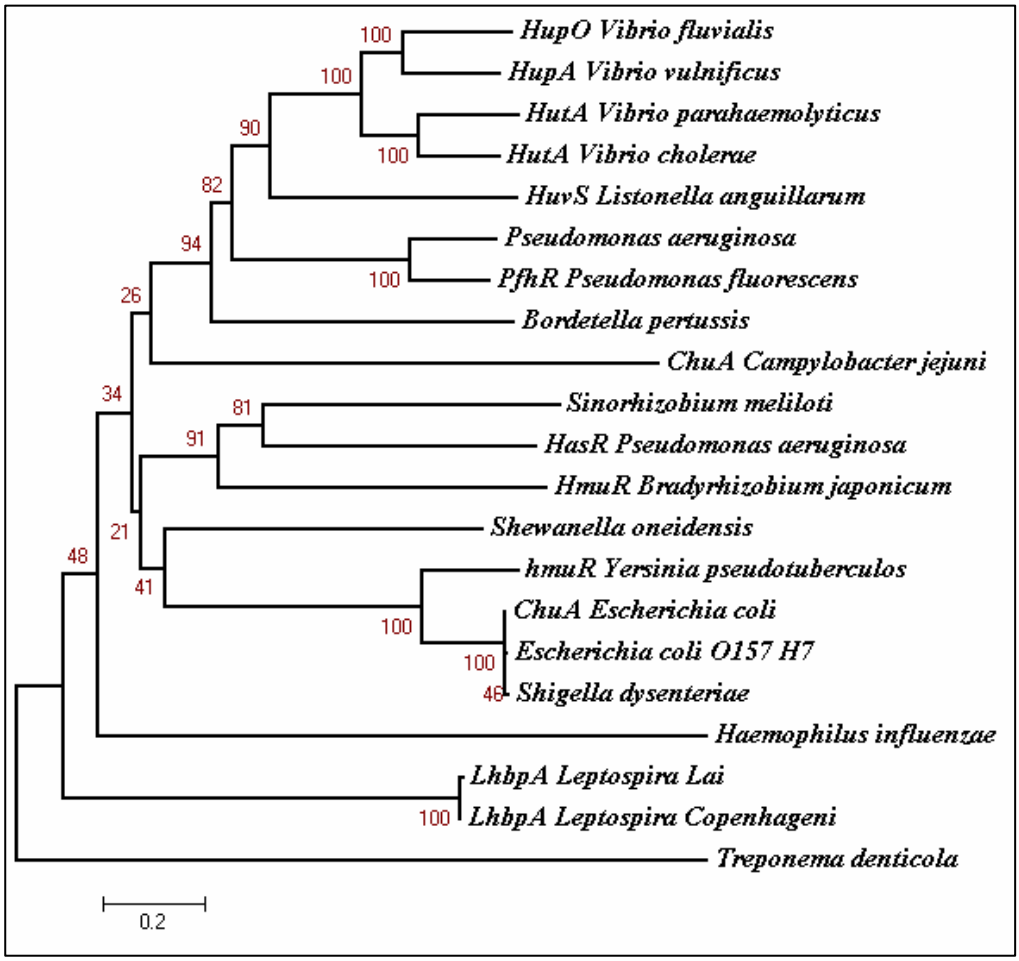


Fig. 31 - Phylogenetic analysis of LhbpA and other bacterial hemin-binding proteins. The tree was constructed using neighbor-joining method with 400 bootstrap replicates, rooted at midpoint and bootstrap values are shown as percentage. A value of 0.1 on the ruler corresponds to a difference of 10% between the two sequences.

The FRAP-NPNL hemin-binding motif was identified in LhbpA (Fig. 32a) and was compared with other bacterial FRAP-NPNL hemin-binding consensus motifs (Bracken *et al*., 1999) (Fig. 32b).

(a)

```
1   METNPILNQL NMFFSFRRFF SSNWIQILFL FLFYVSIQAQ DENIKTENKV IQKESPNTTT
71  TDSKKDNGNG QNGQIVPEES QIVVTGSRGE RRLKDSTVAT EVISRKKIEA SGARNAAEVL
121 ETQLGIDVVP FFGGSRVRML GLDSQYVLIL IDGERISGRL NNAVDLSRFK VQNIERIEIV
181 KGASSALYGA DAIGGVINLI TREADKKLSY EMRTTYGNGS RKNFNTEGEF NTTANMGFRN
241 EYVSGAVSAG YNKNPGYRLV PNSQATTGNA YQDLNTGINL TFNPDGKFKG KTRILYQHRD
301 QNGVDVTQSK AVFDRNNKTH DFLATGSLEY GFGKRNLISF RGNISKWENK YYNNQRGSDE
361 LDVKQLNSEL TSQGTVQLDM EASEKHFITV GAESFANELE SDRLQSRYVY RTRKAVFFQD
421 EWTVSRSPRI RVVPGVRYDD DSQFGNQTTP KLAARYDIFQ NLVWRASYGR GFRPPSFQEL
481 YLRFENPAVG YVVEGNPNLK PERSITINSD LEYSPFSFLT FSLSVYRNDI INLIQYKFDS
541 NKGREFAEFQ LQNIAKAYTR GGEFGVQYRF LKYFTLELGY NHTDTRDLSS DRPLEGRALH
601 QASANFIYNS PGGFQFNLRG KHLDKRPFYS STNNLSAAGQ DYIPSEVKLN ENPPVIYGKP
661 FTILNVRIEQ KFFNKHFALF LGVDNLLNQY ELAYNPTRPR FYYGGFSAQF
```

(b)

		FRAP box	NPNL box	
FHupO	456	SQGFRAPTFSELYYVYAGGCYYGFCYENIPNPDLKSEESISYELGYRH		506
AHuvA	472	SQGFRAPNFTELYYTYDN---IAHRYVNDPNPYLKSETSLAYELGYRH		516
VHupA	469	SQGFRAPSFNELYYTYDN---PGHGYTNRPNPNLESEKSLSYELGYRH		513
PHutA	454	SQGFRAPDFQELYYSFGN---PMHGYIFKPNPDLKAEDSISYEFGWRH		498
CHutA	462	SQGFRAPDFQELYYSFGN---PAHGYVFKPNPNLEAEDSVSYELGWRY		506

Fig. 32 - FRAP-NPNL motif involved in hemin binding. Panel (a) shows the FRAP-NPNL motif in LhbpA and Panel (b) shows the FRAP-NPNL motif in other bacterial hemin-receptors

Fig. 33 shows the neighborhood genes of *lhbpA*, many of which are annotated as hypothetical proteins. Notable, however is the presence of LB186 encoding heme oxygenase.

Gene	Gene length	Location (kb)	Description
LB182	1570	177.00	conserved hypothetical protein
LB183	393	179.45	transcriptional regulator (Fur family)
LB184	141	180.59	hypothetical protein
LB185	111	181.09	hypothetical protein
LB186	670	181.53	heme oxygenase
LB187	1209	182.18	conserved hypothetical protein
LB188	138	183.36	hypothetical protein
LB190	531	183.57	hypothetical protein
LB189	108	184.10	hypothetical protein
LB191	2133	185.00	putative Ton-B dependant outer membrane protein
LB192	411	187.11	conserved hypothetical protein
LB193	111	187.72	hypothetical protein
LB194	579	187.83	unknown protein confirmed by proteomics
LB195	129	188.74	hypothetical protein
LB196	528	189.08	putative LRR repeat family protein
LB197	774	189.62	conserved hypothetical protein
LB198	306	190.81	conserved hypothetical protein
LB199	1356	191.22	putative outer membrane protein
LB200	126	192.68	hypothetical protein
lon A	1623	192.84	ATP-dependant protease LA, putative

Fig. 33 - Neighboring genes of *lhbpA* (lb191)

4. 7. 1. Experimental evidences to show LhbpA is a hemin-binding protein

4. 7. 1. 1. Affinity chromatography using hemin-agarose beads: The binding of recombinant LhbpA to hemin was assessed using hemin-agarose beads (Sigma). Both the induced *E. coli* whole cell extract and the Ni-NTA purified rLhbpA, incubated independently with 100 µl of hemin-agarose beads, showed a single band of molecular size 81 kDa (Fig. 34) upon SDS-PAGE analysis.

4. 7. 1. 2. Assay of heme-dependent peroxidase activity: Based on the intrinsic peroxidase activity of hemin, standard curve was drawn (shown as inset Fig. 35); the amount of hemin bound by varying concentration of rLhbpA was calculated in terms of the peroxidase activity.

4. 7. 1. 3. Spectrofluorimetric analysis for demonstrating the conformational change in LhbpA upon ligand binding: Spectrofluorimetric analysis provided confirmatory evidence for the binding of hemin by rLhbpA (Fig. 36a). When the pure protein was excited at the excitation wavelength of tryptophan (295 nm), a single emission peak at 388 nm was seen. Upon addition of the ligand (hemin), the intensity of the emitted light decreased and with increasing ligand addition, a spectral shift to light of shorter wavelength (approximately 370 nm) was noted.

The binding curve of rLhbpA with hemin (Fig. 36b, inset) drawn by using the fluorescence spectrum data, displayed saturation behavior, implying that the binding occurred at a specific binding site on the protein. The binding capacity of rLhbpA with hemin is calculated as 17.33 mM. The association constant (K_a) of hemin binding was calculated as 6.42×10^4 M^{-1} by using the theoretical fit shown as a Scatchard plot (Fig. 36b) (Scatchard, 1949).

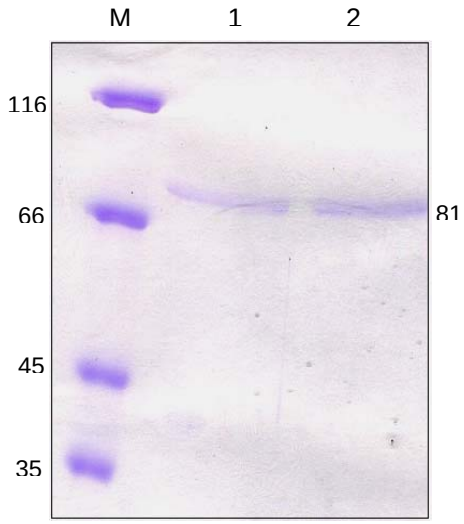


Fig. 34 - Hemin-agarose affinity binding by rLhbpA. The 81 kDa protein eluted out from hemin-agarose when incubated with Ni-NTA column purified rLhbpA (lane 1) and the whole cell sonicate of IPTG-induced recombinant *E. coli* (lane 2) respectively. Lane M - Protein molecular weight marker.

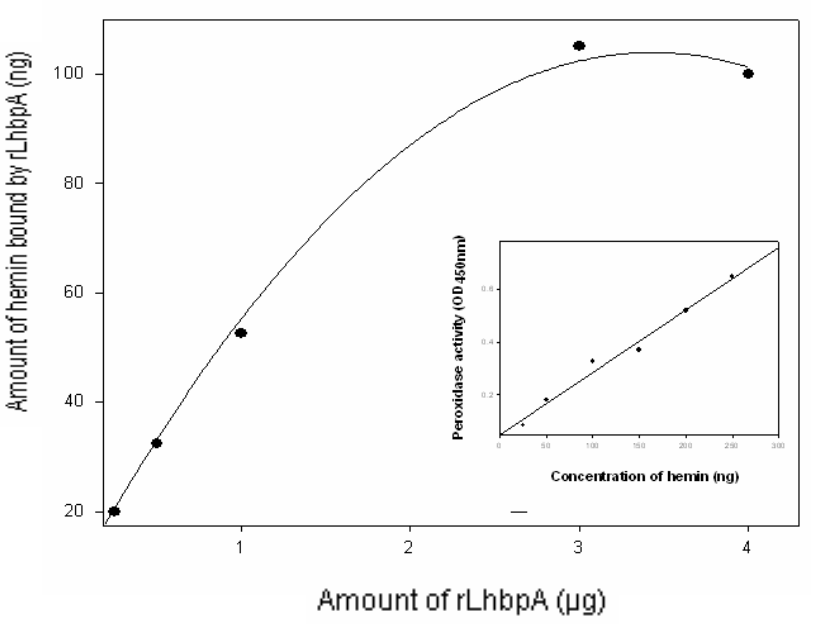


Fig. 35 - Dose dependent binding of hemin by rLhbpA. The amount of hemin bound to rLhbpA was calculated in terms of the peroxidase activity exhibited by rLhbpA upon binding of hemin. The inset shows the standard graph for the peroxidase activity of hemin.

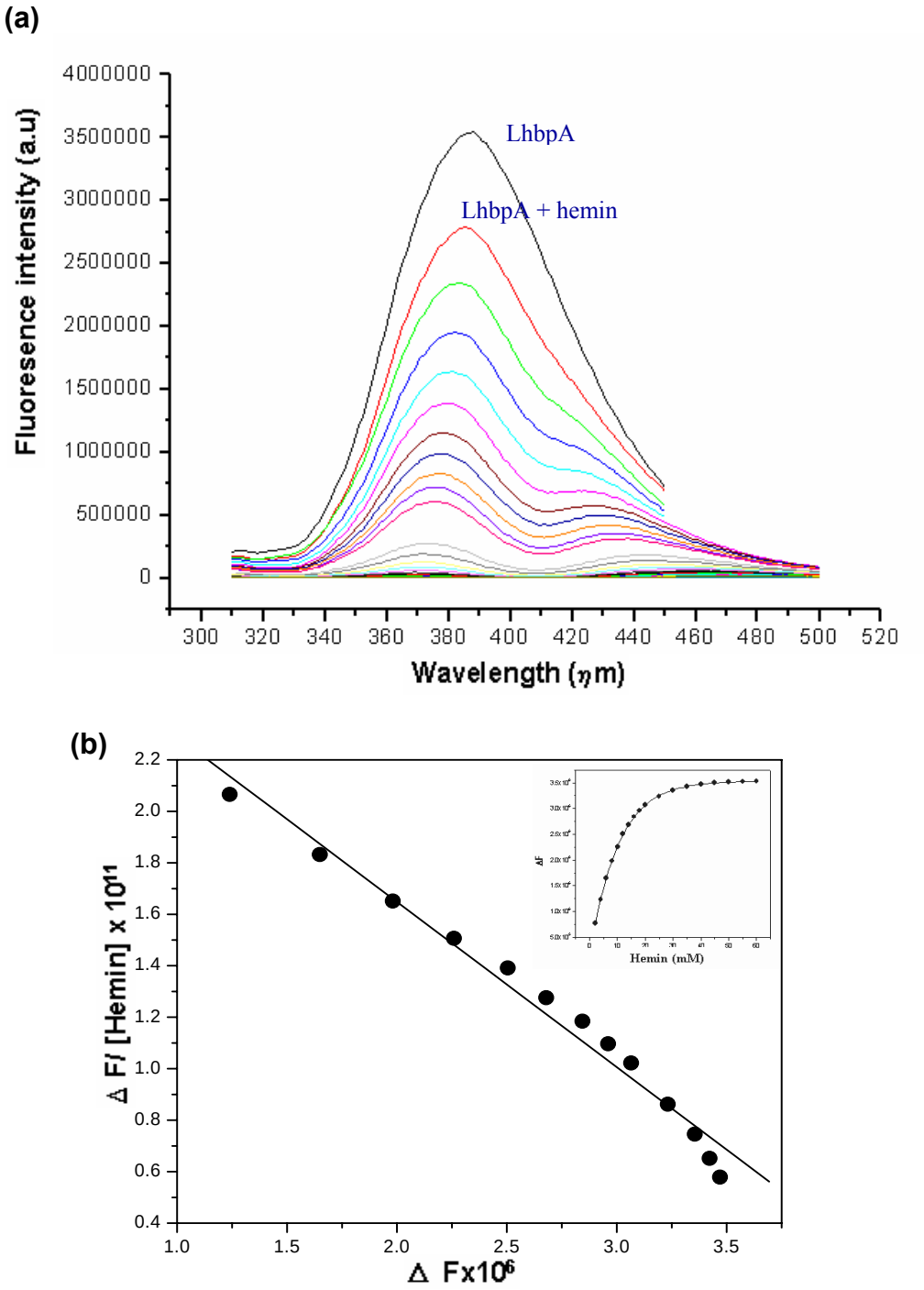


Fig. 36- Spectrofluorimetric analysis for demonstrating conformational change in LhbpA upon ligand binding. Panel (a) shows the fluorescence emission spectra of rLhbpA upon addition of hemin. Panel (b) shows the Scatchard plot for binding of hemin to rLhbpA. The inset shows the decrease of fluorescence intensity as a function of hemin concentration.

4. 8. Iron-regulated expression of LhbpA

4. 8. 1. Presence of Fur box and Fur regulator gene: The *fur* gene (LB183) is located in the neighborhood of the LhbpA (Fig. 33). Promoter analysis using the *E.coli* Fur box consensus sequence has shown two Fur-binding regions upstream of the *lhbpA* gene (Fig. 37).

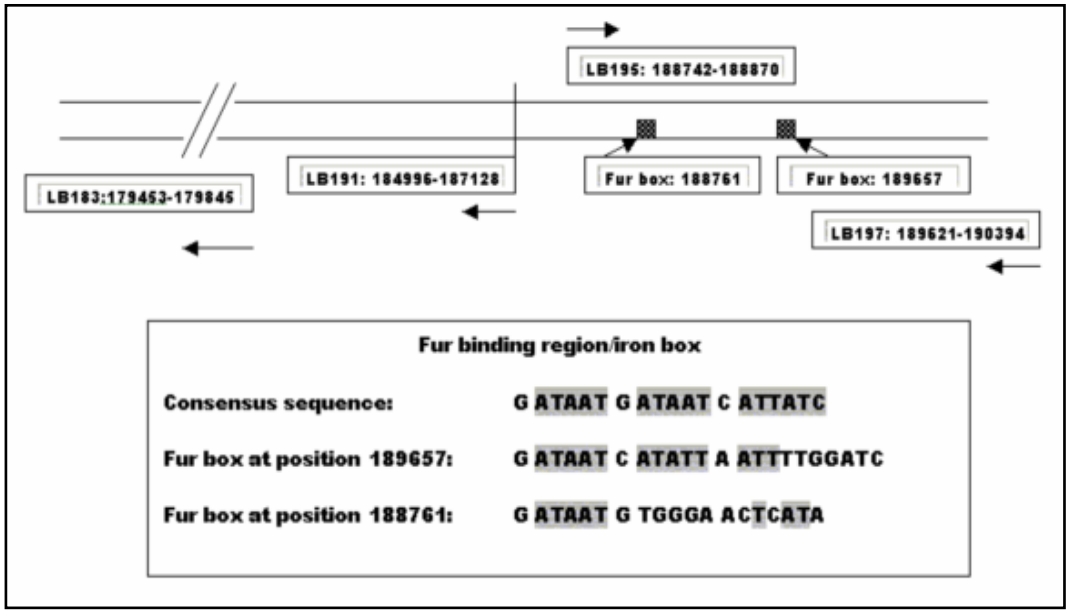


Fig. 37 – Location of two Fur boxes upstream of *lhbpA*. Two Fur boxes are located upstream of LB191, one at -1633 and the second at - 2529 respectively (relative positions in the genome being 188761 and 189657 respectively).

4. 8. 2. Agglutination of low iron *L. interrogans* serovar Lai strain Lai with anti-LhbpA antibodies: As LhbpA is a TonB-dependant outer membrane receptor protein, we studied the agglutination of the organism in the presence of anti- LhbpA antibodies to establish if LhbpA was a surface exposed protein. The organisms were grown under high iron and low iron conditions and after optimization of their cell densities, they were incubated with anti-LhbpA antibodies. The agglutination was notable with low

iron organisms that were incubated at 37°C, (as compared to the cells grown at 30°C) with no agglutination in high iron organisms (Fig. 38).

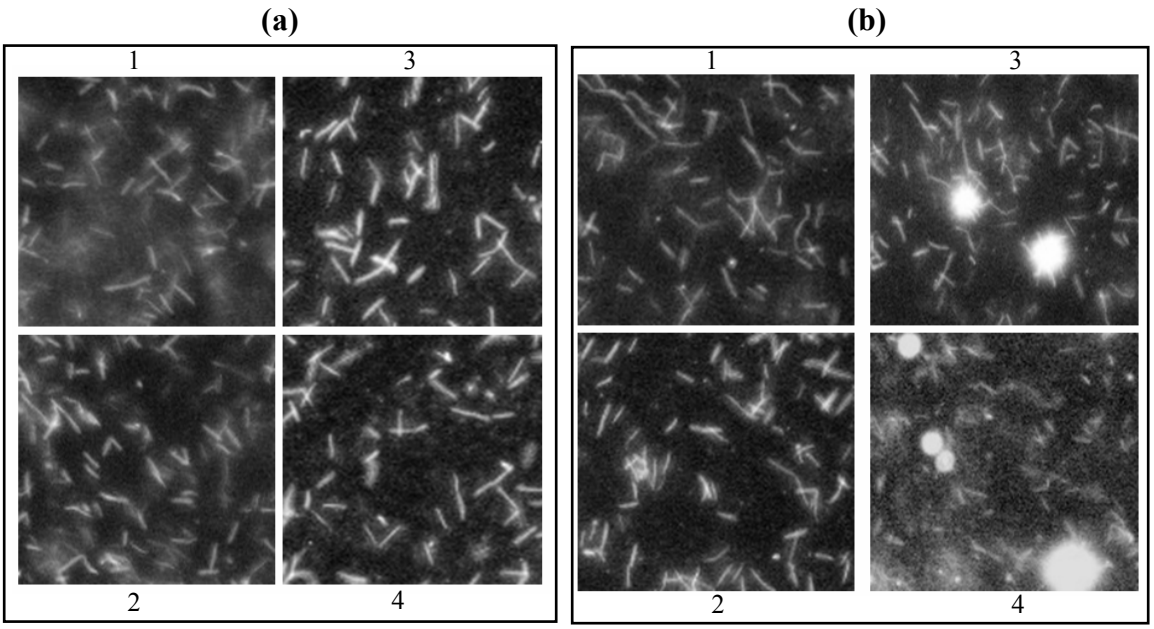


Fig. 38 - Agglutination of iron-limited organisms of *L. interrogans* serovar Lai by anti-LhbpA antibodies. Panel (a) and (b) represent high and low iron organisms. Each panel represents live organisms (1), live organisms incubated with pre-immune serum (2), live organisms incubated with anti-LhbpA antibodies added at dilutions of 1:50 (3) and 1:100 (4) respectively.

The agglutination was observed within 15 min upon addition of serum and after 2 h of incubation, notable lowering of cell numbers was noted. No agglutination was observed in low iron organisms incubated with pre-immune serum (negative control).

4. 8. 3. Evolutionary relationship of LhbpA with other iron-regulated virulence proteins: LhbpA showed phylogenetic relationship with many bacterial iron-regulated virulence proteins especially with the *Campylobacter coli and Pseudomonas fluorescens* (Fig. 39).

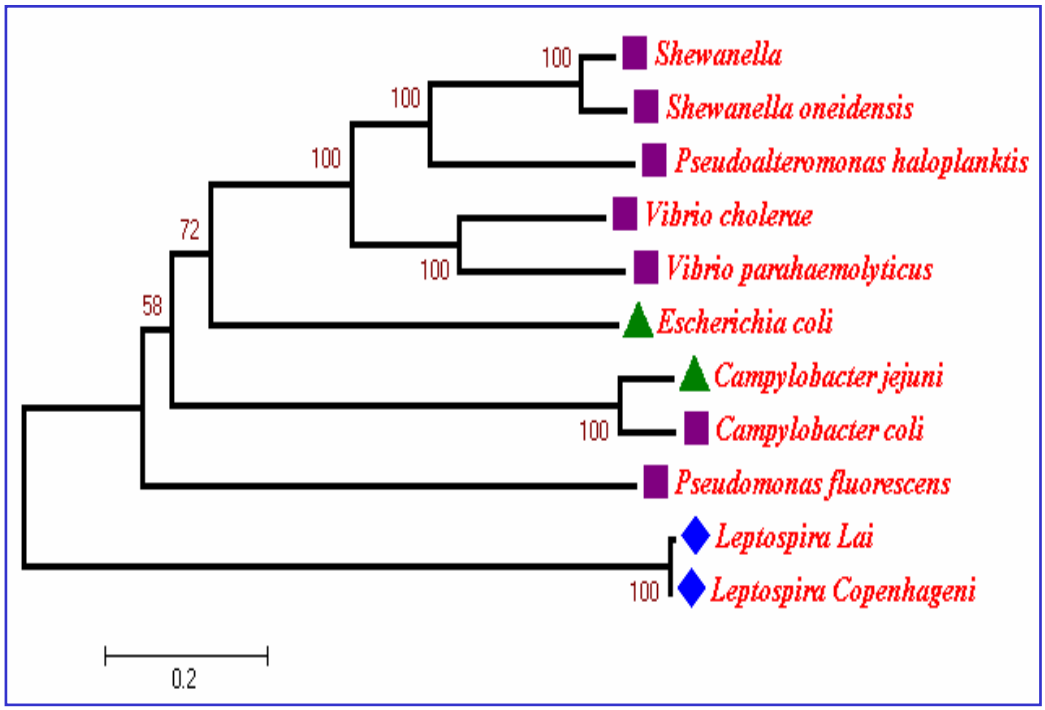


Fig. 39 - Phylogenetic analysis of LhbpA and other bacterial iron-regulated virulence proteins. The tree was constructed using neighbor-joining method with 400 bootstrap replicates, rooted at midpoint and bootstrap values are shown as percentage. A value of 0.1 on the ruler corresponds to a difference of 10% between the two sequences.

5. PART-II - STUDIES ON IRON LEVELS AND SPHINGOMYELINASES

5. 1. *In Silico* analysis of hemolysin genes of *L. interrogans* serovar Lai strain Lai

The genome of *L. interrogans* serovar Lai strain Lai has about 10 different genes putatively encoding hemolysin proteins as listed in Table 5.

Table 5- Hemolysin genes of *Leptospira interrogans* serovar *Lai*

S. No	Gene Name	Locus Tag	Gene length (bp)	MW of the expressed protein (kDa)
1	Sphingomyelinase C precursor *sph1*	LA1027	1793	68.19
2	Sphingomyelinase C precursor *sph2*	LA1029	1871	71.03
3	Sphingomyelinase C precursor *sph3*	LA4004	1676	65.33
4	Hemolytic protein-like protein *sph4*	LA3050	719	27.92
5	Sphingomyelinase C precursor *sphH*	LA3540	1664	64.43
6	Hemolysin-like protein (*hlyC*)	LA3937	1334	50.43
7	TPR repeat-containing proteins (*hlyX*)	LA0378	1178	44.95
8	Hemolysin (*tlyA*)	LA0327	830	31.67
9	Hypothetical protein (*hlpA*)	LA1650	941	36.53
10	Hypothetical UPF0161 protein	LA0177	232	8.5

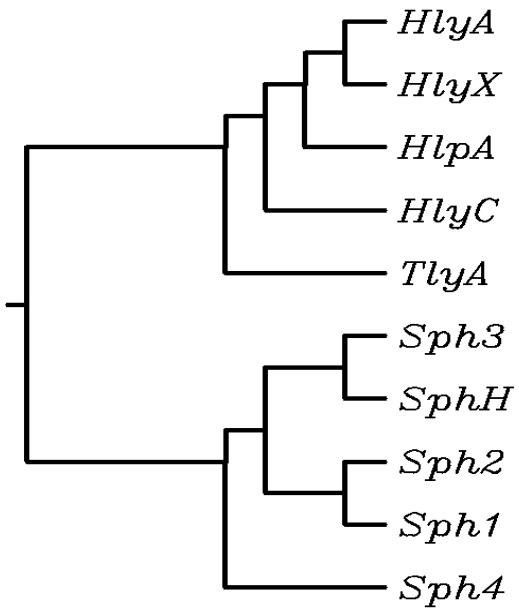
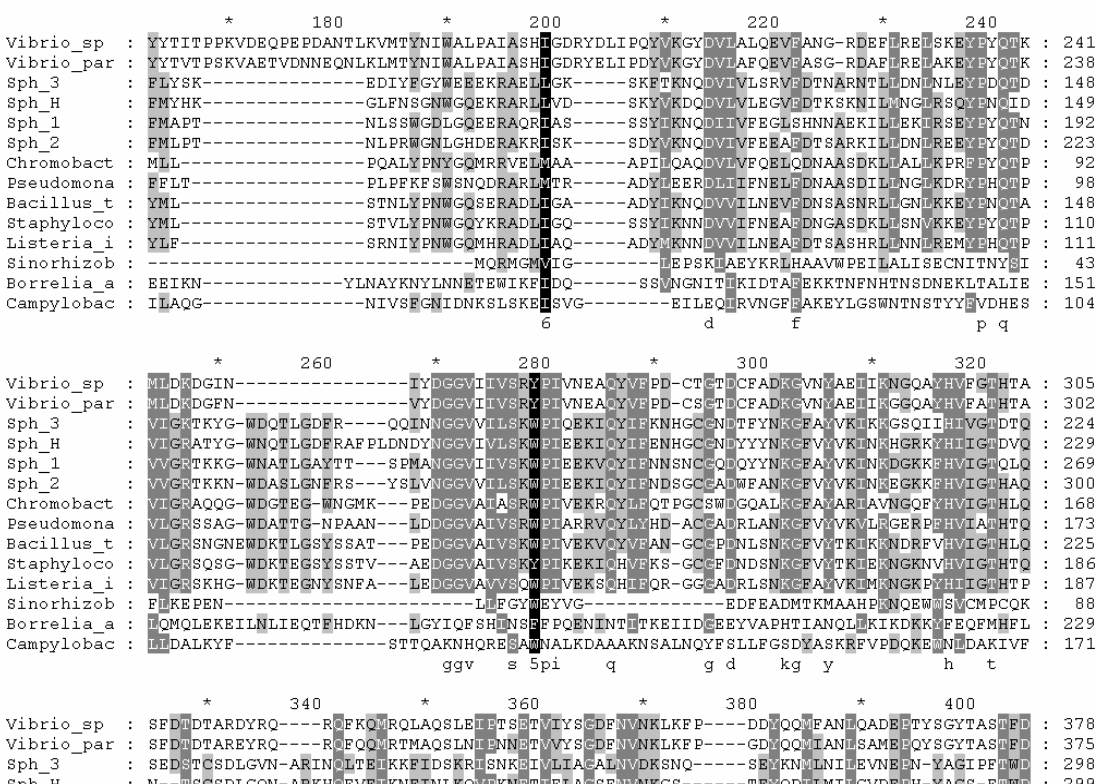


Fig. 40 – Phylogenetic analysis of hemolysins of *Leptospira* spp. The sphingomyelinase-like hemolysins (Sph1, Sph2, Sph3, Sph4 and SphH) form separate rooted branch from that of non-sphingomyelinase-like hemolysins (HlyA, HlyX, HlyC, HlpA and TlyA).

Phylogenetic analysis and amino acid sequence alignment was performed on the four sphingomyelinase-like hemolysin (sph) proteins of *Leptospira* spp namely Sph1, Sph2, Sph3 and SphH (LA1027, LA1029, LA4004, LA3540) and related proteins of several bacteria (Fig. 40, Fig. 41a & Fig. 41b). The leptospiral Sph proteins of *L. interrogans* made a separate rooted branch in the phylogenetic tree. They shared highest homology among themselves and were different from other bacterial hemolysins, showing relatively close evolutionary relationship with Sph of *Vibrio* spp. and *Pseudomonas* spp.

(a)


Vibrio_sp
Vibrio_par
Sph_3
Sph_H
Sph_1
Sph_2
Chromobact
Pseudomona
Bacillus_t
Staphyloco
Listeria_i
Sinorhizob
Borrelia_a
Campylobac

(b)

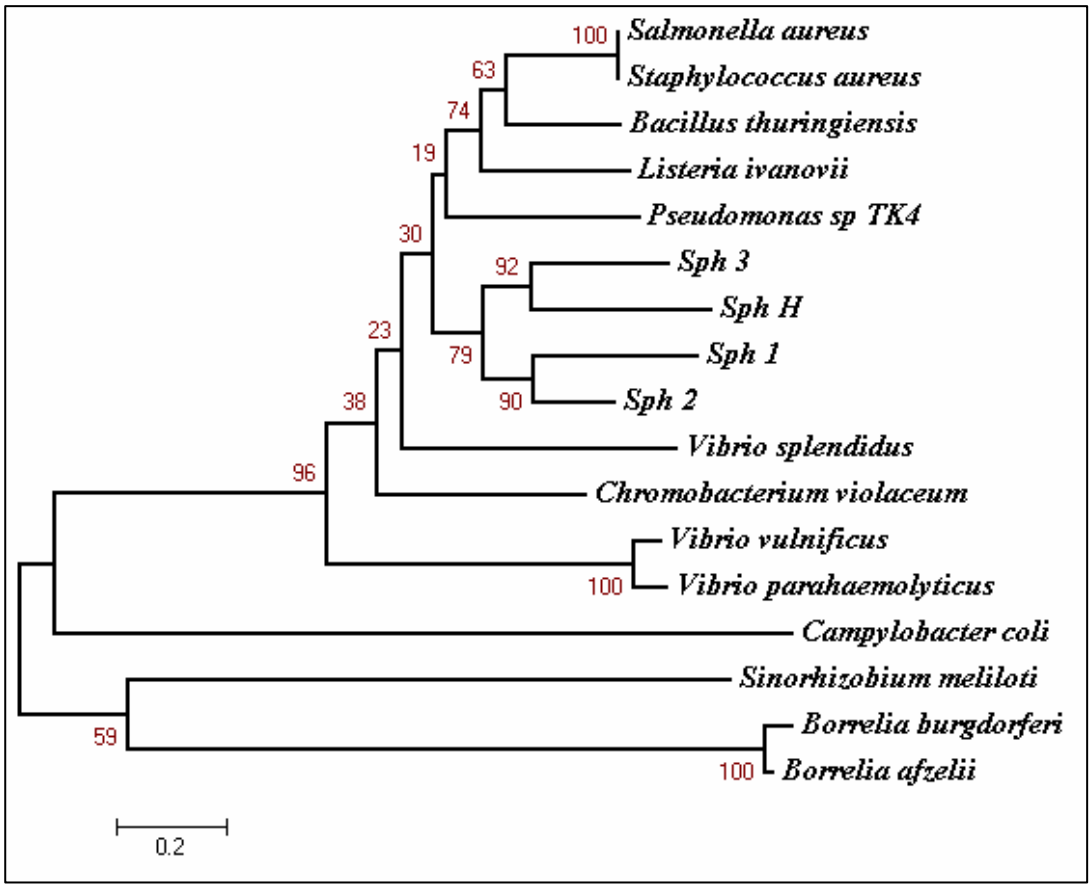


Fig. 41a – Multiple sequence alignment of deduced amino acid sequences of Sphingomyelinases (Sph) of *L. interrogans* serovar Lai and other bacterial hemolysins. The alignment and shading of amino acid residues was done using Gene Doc software. Identical amino acid residues are shown on a black background and the match occurring within the same column is shown with a gray background. Dash (-) indicate sequence gaps to optimize the alignment.

Fig. 41b - Phylogenetic analysis of Sph proteins. The tree was constructed using neighbor-joining method with 400 bootstrap replicates, rooted at midpoint and bootstrap values are shown as percentage. A value of 0.1 on the ruler corresponds to a difference of 10% between the two sequences.

5. 2. Cloning and expression studies

The 638 bp region with the highest homology between *sph*1, *sph*2, *sph*3 and *sphH* was identified using ClustalW software (Fig. 42).

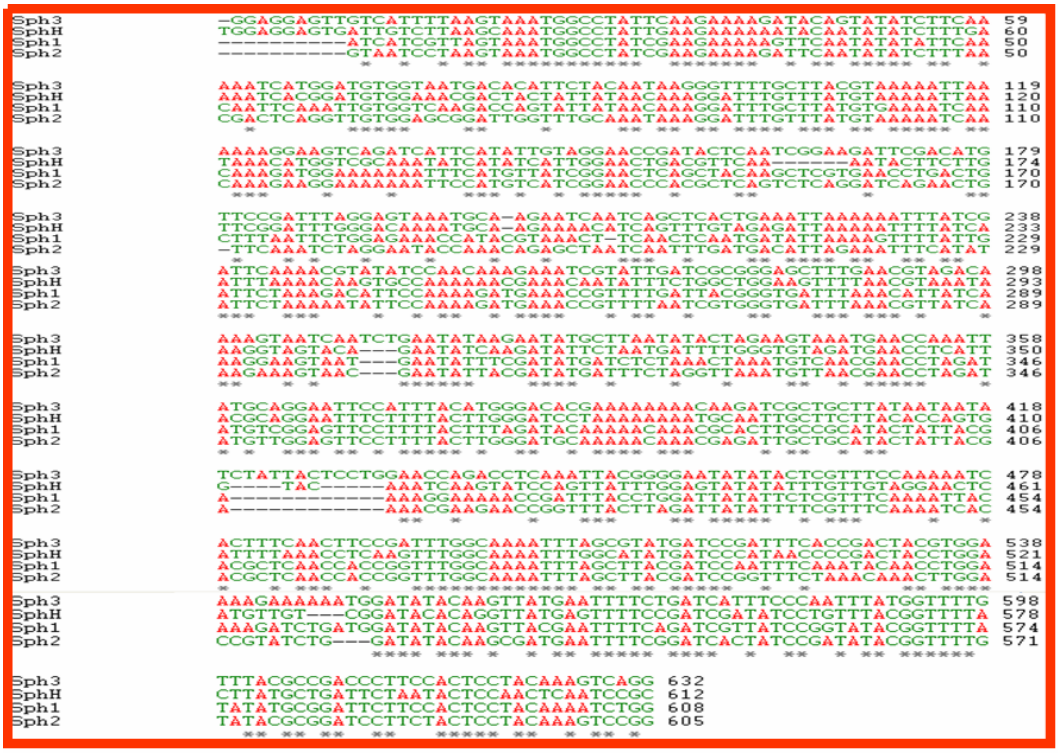


Fig. 42 – Nucleotide sequence of the 638 bp region common to *sph*1, *sph*2, *sph*3 and *sphH*

Specific primers S1 & S2 were designed (Table 4) to amplify the 638 bp region to give sph_{638}, which was digested with *NdeI* and *HindIII* and ligated into compatible restriction sites of pET-28(a) vector. The recombinant was transformed into DH5α and BL21 (DE3) pLys(S) cells for sequencing and expression studies respectively (Fig. 43). Positive clones were identified by the 638 bp product seen upon colony PCR. One of the positive clones was subjected to sequencing analysis using gene-specific forward and reverse primers to confirm the insert (Fig. 44a & Fig. 44b).

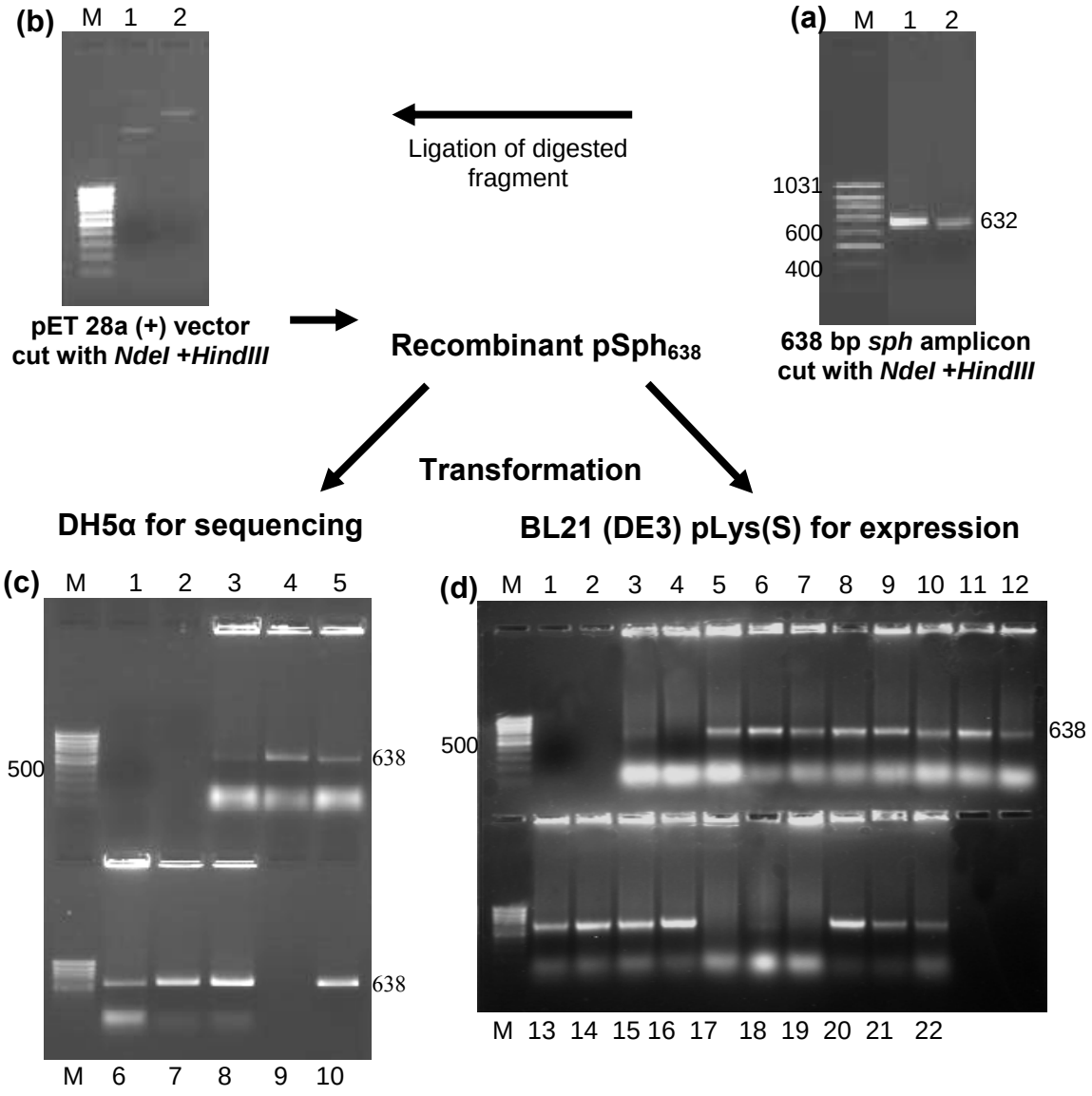


Fig. 43 - Cloning strategy for *sph*$_{638}$ amplicon (Panel (a), lane 1) was restriction digested using *NdeI* and *HindIII* enzyme (Panel (a), lane 2) and was ligated into pET 28a (+) vector (Panel (b), lane 1), restricted digested with *NdeI* and *HindIII* enzyme (Panel (b), lane 2). The recombinant pET 28a (+) plasmid (pSph$_{638}$) was transformed into DH5α for sequencing (Panel c) and BL21 DE3 pLys(S) cells for expression studies (Panel d). Positive clones were identified by the 638 bp product seen upon colony PCR (Panels c & d). Lane M represents DNA molecular weight marker

Fig. 44 - Sequence analysis of the *sph*$_{638}$ insert. The sequenced DNA region of *sph*$_{638}$ using gene-specific forward (Panel a) and reverse primer (Panel b) is compared with the sequence in the genome (Panel c and Panel d respectively) and is represented as bar diagram.

Expression of the 33 kDa recombinant protein in pET 28a (+) was confirmed by SDS-PAGE analysis of the cell free sonicate of IPTG-induced clones (Fig. 45a). The $rSph_{638}$ protein was expressed in the insoluble fraction as inclusion bodies (Fig. 45b). The pellet was solubilized by 2% SDS and subjected to SDS-PAGE. $rSph_{638}$ was purified by eluting the 33 kDa band from the gel (Fig. 45c). The $rSph_{638}$ was recognized by His-tag antibodies and anti-$rSph_{638}$ antibodies (Fig. 46).

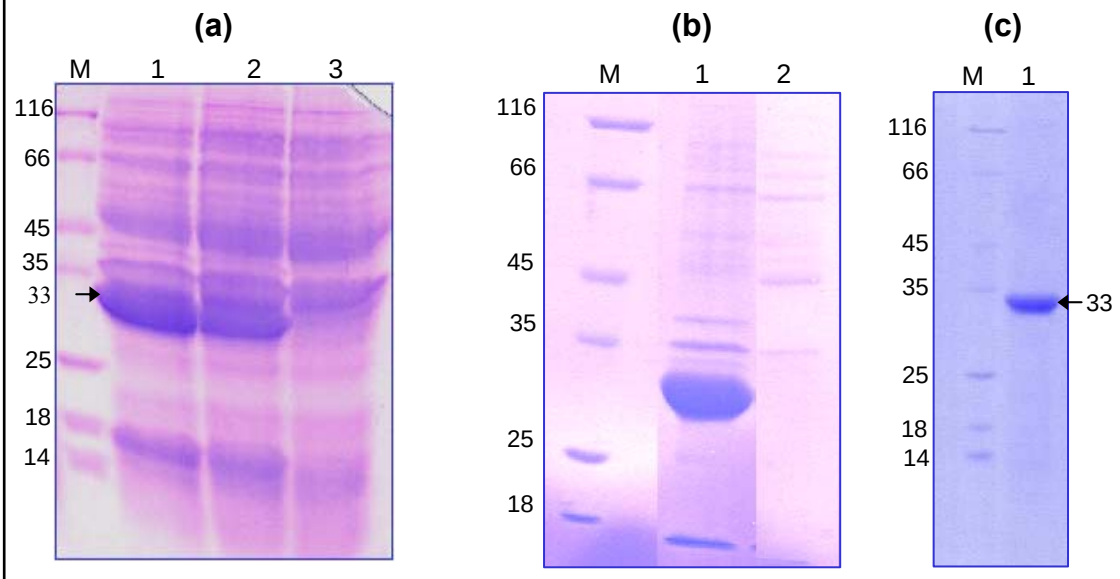


Fig. 45 - Expression and purification of $rSph_{638}$ protein. Panel (a) shows the expression of $rSph_{638}$ at 2 h (lane 2) and 3 h (lane 1) of IPTG induction and is compared with the proteins expressed by the un-induced *E. coli* cells (lane 3). Panel (b) shows the expressed protein in the insoluble fraction after sonication (lane 1) and no traces of it was found in the soluble fraction (lane 2). Panel (c), lane 1 represents the purified rSph-638 by gel elution technique. Lane M is the protein molecular weight marker.

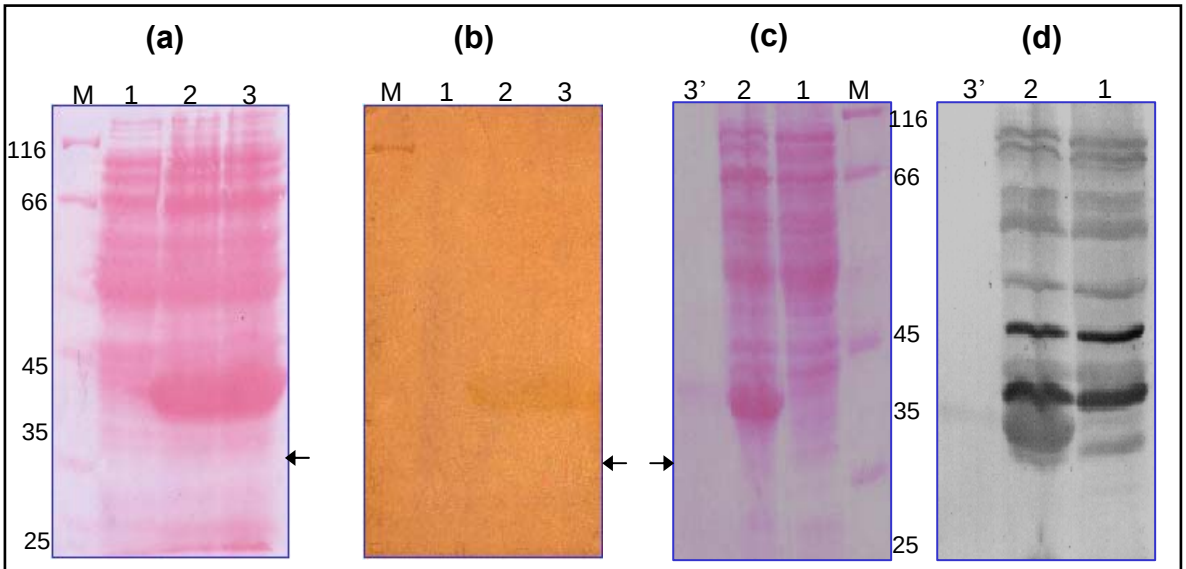


Fig. 46 - Immunoreactivity of rSph$_{638}$ with anti His-tag (Panels a & b) and anti-rSph$_{638}$ antibodies (Panels c & d). Panel (a) and (c) shows the Ponceau stained nitrocellulose membranes and the Panel (b) and (d) shows the corresponding developed blots. The different lanes represents proteins expressed by un-induced *E. coli* cells (lane 1), IPTG induced *E. coli* cells (lanes 2 & 3) and purified rSph$_{638}$ (lane 3'). Lane M is the protein molecular weight marker.

5. 3. Iron-regulated expression of sphingomyelinase-like hemolysin

5. 3. 1. Promoter analysis of sphingomyelinase-like hemolysin genes: Promoter analysis done by BPROM program of LA1027, LA1029 and LA3540 revealed the presence of Fur box and the program showed that the Fur sequence is more closely related to that in *Pseudomonas aeruginosa* (Fig. 47).

5. 3. 2. Expression of sphingomyelinase-like hemolysins under conditions of iron limitation in *L. borgpetersenii* serovar Ballum strain MUS127: Sph expression was shown to be controlled by iron conditions by i) RT-PCR analysis ii) Hemolytic assay and iii) Western blot analysis.

5. 3. 2. 1. RT-PCR analysis: RT-PCR analysis in *L. borgpetersenii* serovar Ballum strain MUS127 using S1 & S2 primers showed sph_{638} transcript was significant in low iron organisms as compared to high iron organisms (Fig. 48).

E. coli -	GATAATGATAATCATTATC
P. aeruginosa -	GATAATAAATCTCATTTCC
Leptospiral *sph1* -	GAAAAT------TCTCTTCAAAATC (88-106 bp upstream of gene)
	GATAACAAGTTTCAATTCA (270-288 bp upstream of gene)
Leptospiral *sph2* -	TAAAATACTATTCATTTTT (431-449 bp upstream of gene)
Leptospiral *sphH* -	GATACTGCGTCTCAGAATC (452-470 bp upstream of gene)

Fig. 47 - Promoter analysis of *sph* genes using BPROM programme. Fur Box was identified upstream of *sph1*, *sph2* and *sphH* and is homologous to Fur box sequence of *Pseudomonas aeruginosa*.

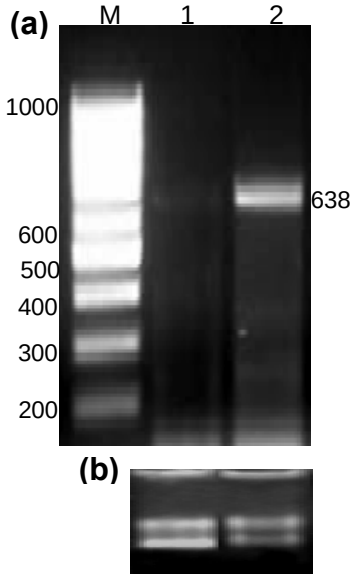


Fig. 48 - RT-PCR analysis of sph_{638} transcript in *L. borgpetersenii serovar* Ballum strain MUS127. The sph_{638} transcript was higher in low iron cells (Panel a, lane 2) as compared to high iron cells (Panel a, lane 1). Panel (b) shows the equivalent levels of RNA in high and low iron organisms. Lane M is the 100 bp DNA marker.

5. 3. 2. 2. Hemolytic assay – activity of hemolysin(s) on sheep erythrocytes: The serovar Ballum was grown under high and low iron conditions, centrifuged to separate the cells and the culture filtrate and assayed for the hemolytic activity in both the whole cell sonicate and culture filtrate. From Fig. 49, it can be seen that under low iron conditions 33% and 8% hemolysis was seen for whole cell sonicate and culture filtrate respectively while under high iron conditions, 18% and 4% were observed for the respective fractions.

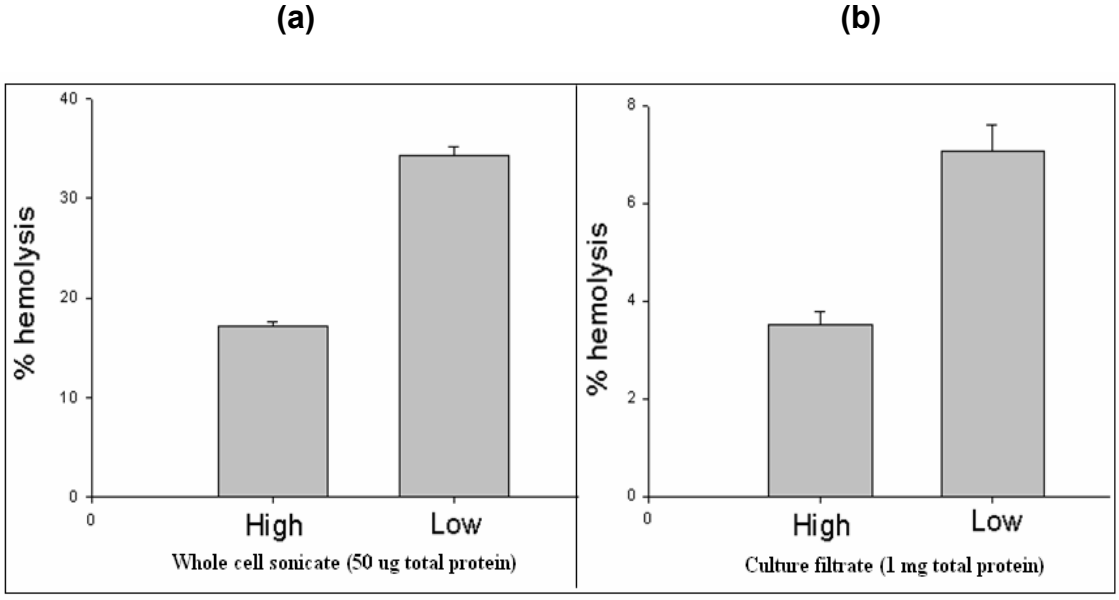


Fig. 49 - Hemolytic assay of *L. borgpetersenii* serovar Ballum strain MUS127 grown under high iron and low iron conditions. Panel (a) represents the hemolytic activity of whole cell sonicate and Panel (b) represents the hemolytic activity of culture filtrate

5. 4. Immunoreactivity of rSph-638 with clinical serum samples (dot-blot assay)

MAT positive serum samples from patients of leptospirosis (Velineni *et al*., 2007) were tested by dot-blot assay for the presence of antibodies against $rSph_{638}$ (Fig. 50a). The intensities of the blots were separately estimated on densitometer and were graphically represented by taking density counts (CNT / mm.sq) on Y-axis and serum samples on X-axis (Fig. 50b). The results obtained were compared with MAT results (Table 6) and it was observed that samples identified as Icterohaemorrhagiae positive by MAT test showed high reactivity with $rSph_{638}$ when compared with that of Autumnalis / Australis / mixed infection; very low reactivity was seen in samples from healthy patients.

Statistical analysis was done using Kappa statistics. The Kappa statistics is a decimal measure of agreement between the two tests especially in the absence of a standard and is defined as Kappa or K. The sensitivity, specificity and accuracy of the dot-blot assay were calculated as 93%, 41% and 80% respectively. The Kappa or K value was calculated to be 97%, which indicates perfect agreement. When the analysis was done with all the samples, the specificity was only 41%; however, when it increased to 60% when only the Icterohaemorrhagiae positive samples by MAT were considered for analysis.

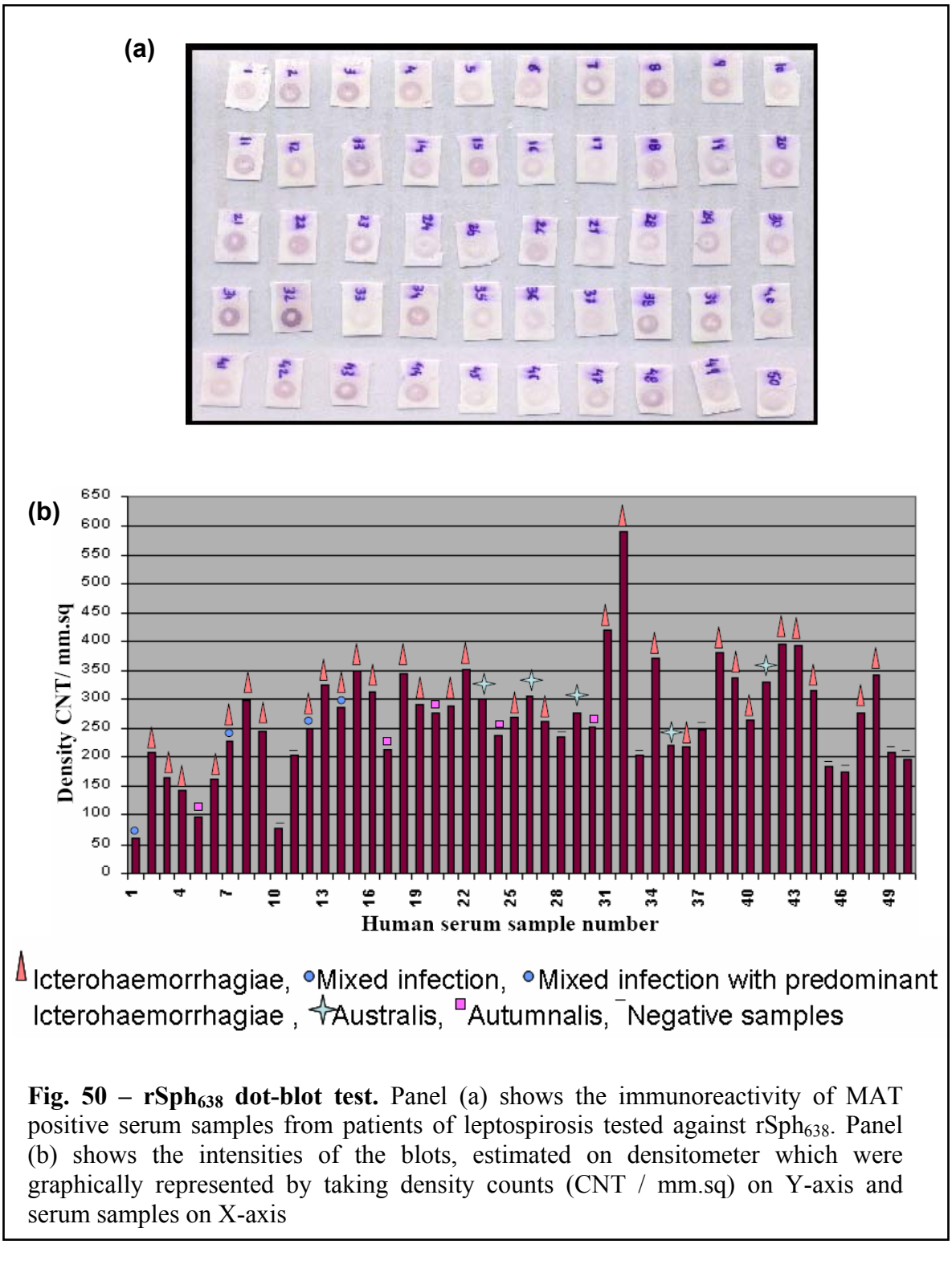


Fig. 50 – rSph$_{638}$ dot-blot test. Panel (a) shows the immunoreactivity of MAT positive serum samples from patients of leptospirosis tested against rSph$_{638}$. Panel (b) shows the intensities of the blots, estimated on densitometer which were graphically represented by taking density counts (CNT / mm.sq) on Y-axis and serum samples on X-axis

Table 6 - Comparison of dot blot analysis using $rSph_{638}$ with MAT

		MAT		Total
		Positive	**Negative**	
Dot-blot analysis	**Positive**	31	2	33
	Negative	7	5	12
Total		38	7	45

Discussion

Pathogens have evolved diverse systems for acquiring iron, 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 and Wooldridge & Williams, 1993). Even though the nutritional requirement for iron by pathogenic *Leptospira* is well known (Faine, 1959), little is understood about the mechanisms of iron acquisition by these organisms. In this study, we first report our observations on the effect of iron limitation in the saprophytic *L. biflexa*, demonstrating siderophore expression and iron-regulated proteins. Subsequently, using a bioinformatic approach we report a TonB-dependant outer membrane protein from the genome of *L. interrogans* serovar Lai (LB191) as a possible hemin receptor called as LhbpA (leptospiral hemin binding protein A). We cloned and expressed the full length LhbpA and using rLhbpA, we provide experimental evidence to prove that this 81 kDa protein is a hemin-binding protein. The iron-regulated expression and the surface localisation of this protein are demonstrated in this study. In the second half of this study, we present our observations on the role of iron on the expression of sphingomyelinases in the serovar Ballum.

Leptospires are fastidious and require specialized media for optimal growth in the laboratory. Traditionally, EMJH medium with commercial enrichment medium containing Tween 80 and BSA are used, as these organisms do not utilize carbohydrates and use lipids as the source of carbon and energy. Due to the complex medium, establishment of conditions of iron limitation was not easy, as normally done for other bacterial systems. Iron chelators were used for effecting iron limitation;

different chelators like EDDA, EDDHPA, 2, 2'-dipyridyl and 10% serum were optimized with the saprophytic *L. biflexa* serovar Patoc strain Patoc I. In addition, iron-limiting media with iron-free chemicals and reagents were used, with the growth carried out in iron-free glassware. The expression of siderophores was assayed using the Chrome Azurol Assay (CAS) (Schwyn and Neilands, 1987). In the CAS agar plate with 0.02 μg / ml of iron, the blue colour of the chrome azurol-iron complex disappeared and the orange colour of the dye was seen concentrated in the cells, while the cells remained colourless in the plate with iron concentration at 4 μg / ml. Iron-regulated proteins were analysed in these organisms. Analysis of the Triton X-114 extracts of the cells grown in the presence of the iron chelators, showed the expression of four iron-regulated proteins of apparent molecular mass 82, 64, 60 and 33 kDa (Sritharan & Asuthkar, 2004). The 82 kDa was observed in the aqueous extract indicating it might be a periplasmic protein, while the other three proteins were seen in both the aqueous and detergent extract. In the organisms grown with iron added at 0.02 μg Fe / ml, the 64, 60 and the 33 kDa proteins were seen. However, their expression could also be seen in cells that were grown with 4 μg Fe / ml. This concentration of iron is still probably not sufficient to completely repress the expression of these proteins. At this concentration of iron, these cells expressed a prominent 24 kDa protein that was down-regulated at 0.02 μg Fe / ml. This protein band was seen in both the aqueous and detergent extracts.

As pathogenic leptospires were found to be sensitive to the addition of chelators, an alternative bioinformatic approach was taken to identify a putative iron-

regulated protein from the genome described for *L. interrogans* serovar Lai (Ren *et al*., 2003). Using this bioinformatic approach, we identified a putative TonB-dependent outer membrane receptor protein (locus tag LB191) from the genome of serovar Lai by homology with the ferric enterobactin receptor FepA of *E. coli*. As our analysis led us to hypothesise this protein as a putative iron-regulated, hemin-binding protein, we initially called it as LEP_IRMP (leptospiral iron-regulated membrane protein); however, in keeping with the standard norms for the nomenclature of proteins, we have re-named this protein as LhbpA (leptospiral hemin-binding protein A). This protein, despite showing low level of similarity (39%) and identity (22%) with FepA of *E. coli*, revealed features of protein folding like other Fe^{3+}-siderophore receptors. Upon homology modeling with Insight II software, using FepA as the reference protein, the 3D structure of LhbpA was found to show the characteristic β -barrel structure with the three domains, namely the β-barrel, plug domain and N-terminal TonB box. The high degree of structural similarity between LhbpA and FepA is evident. The β-barrel domain, forming the trans-membrane part of the receptor consists of 22 anti-parallel strands with large loops extending towards the extracellular side and short periplasmic turns facing the periplasmic side. As expected, there were striking differences in the sequence between LhbpA and FepA in the extracellular loops, reflecting their ligand specificity. The ferric siderophore receptors FepA (Rutz *et al.,* 1992), FhuA (Locher *et al.*, 1998) and FecA (Ferguson *et al.,* 2002) of *E. coli* also differ in these sequences reflecting ligand specificity for ferric enterobactin, ferrichrome and ferric citrate respectively. The plug domain of LhbpA, consisting of

six helices and six β strands in the N-terminal region shares a high degree of homology with FepA protein. The TonB box in LhbpA, represented by the sequence ESQIVVTGS was located between amino acids 79-87 and it differed from the consensus sequence in the 3rd amino acid Q.

BLASTP analysis of microbial genomes showed that LhbpA shared strong homology with ferric siderophore protein receptors / heme-binding proteins / colicin receptors / Vitamin B12 receptors of several Gram-negative bacteria; this is not an unexpected observation, as iron transport systems are known to facilitate the transport of colicins and vitamin B12 in several Gram-negative bacteria. In addition to the strong structural similarity of LhbpA with other ferric-siderophore receptors, the presence of the *fur* gene (LB183) and the Fur box in the vicinity of the *lhbpA* gene indicated it is an iron-regulated protein. The gene encoding heme oxygenase (LB186) was located in the neighbourhood of *lhbpA* and the amino acid sequence of LhbpA showed the FRAP-NPNL motif that is associated with heme binding. Based on the hypothesis that LhbpA was a putative iron-regulated, hemin-binding protein, experiments were designed to prove our hypothesis.

The full length 2148 bp *lhbpA* gene was PCR amplified, cloned and expressed in the expression vector pET 28a (+). Some bacterial outer membrane proteins are toxic when over-expressed in *E. coli* (Xu *et al*., 2001), the toxicity being ascribed to the 5' region of the gene that encodes the signal peptide. We found this to be true for the full-length LhbpA, with the formation of small colonies and the low level of expression of the 81 kDa rLhbpA protein. Three lines of experimental evidence have

been provided to confirm that LhbpA is a hemin-binding protein. They include affinity binding to hemin-agarose beads, spectrophotometric and spectrofluorimetric quantitation of hemin binding. The 81 kDa LhbpA protein, purified by Ni-NTA column bound hemin-agarose beads as analysed by SDS-PAGE. When whole cell sonicates of the IPTG-induced recombinant clone was added to hemin-agarose beads, the 81 kDa band eluted out as a single band without any contaminating proteins. Spectrophotometric assay of the peroxidase activity of the hemin bound by LhbpA and the saturation curve seen in the fluorescence spectrum data show that LhbpA bound hemin in a dose-dependant manner. The quenching of the emission spectrum, with maximal intensity at 388 nm and the spectral shift to a lower wavelength seen in the spectrofluorimetric analysis indicates that hemin bound a specific site on the protein, possibly involving a tryptophan residue.

LhpbA probably transports hemin by a mechanism similar to other Fe^{3+}-siderophores / hemin receptors (Braun & Braun, 2002). The receptor, upon ligand binding interacts with the TonB protein via the TonB box. The energy for this process is derived by the TonB system consisting of the TonB, ExbB, ExbD proteins that mediate the transfer of the proton motive force of the cytoplasmic membrane to the outer membrane receptors. The Fe^{3+} complexes are internalised via specific proteins in the periplasm, which deliver the Fe^{3+} and the Fe^{3+} compounds to the ABC transporters in the cytoplasmic membrane. Thus, LhbpA is a hemin-receptor that functions like other TonB-dependant outer membrane Fe^{3+} transporters, mediating the uptake of Fe^{3+} via hemin. However, further studies are required to understand if the entire molecule is

internalised or the Fe^{3+}, released at the cell surface is taken up via this protein. Though it has been reported that saprophytic and pathogenic species of *Leptospira* are capable of both *de novo* synthesis and uptake of heme (Guegan *et al*., 2003), the specific receptors and mechanism of transport is not delineated.

Iron-regulated expression in Gram-negative bacteria is generally under the control of the Fur protein, which represses the transcription of iron-regulated promoters in response to an increasing intracellular Fe^{2+} concentration. Fur, not only regulates the genes involved in the biosynthesis and uptake of siderophores (Guerinot, 1994), but also a variety of iron-dependent cellular processes, such as the oxidative-stress response (Tardet & Touati, 1993) and the expression of virulence-associated genes (Salyers & Whitt, 1994; Sritharan, 2000 and 2006). The presence of two Fur boxes in the upstream region of *lhbpA* and the fur regulator gene (LB183) in the neighborhood indicate the possible regulation of *lhbpA* expression by iron. In bacterial systems, chain of functionally related genes transcribed in the same direction and with small intergenic distances are assumed to form a candidate operon, and a candidate site upstream of this operon is accepted as the one regulating all genes in the chain. In *E. coli*, the iron uptake systems including aerobactin biosynthesis and uptake system *iucABCDiutA*, the ferrichrome-uptake system *fhuACDB* (Fecker & Braun, 1983), the uptake / sensory system *fecIRABCDE* for the uptake of ferri-dicitrate complexes (van Hove *et al*., 1990) and large locus-containing genes for enterobactin biosynthesis (*entABCDEFG*) and uptake (*fepABCDEG*) are organized into five small operons (Grunberg-Manago, 1996). The *fur* gene (LB183), heme oxygenase (LB196) and

many hypothetical proteins, whose functions are not yet identified, are present in the vicinity of *lhbpA* gene. These genes together with *lhbpA*, thought to be involved in iron / heme uptake could be a part of an operon whose expression is controlled by Fur protein which binds to the upstream Fur box region

LhbpA is expressed on the cell surface of *L. interrogans*, maintained under conditions of iron limitation and at 37ºC. Growth of pathogenic leptospires under conditions of iron limitation proved to be difficult, as they failed to grow in low iron media and they were sensitive to iron chelators like 2, 2'-dipyridyl. The optimization of the growth conditions was simultaneously carried out as a separate doctoral study. These organisms were however used in this study to show the agglutination of live leptospires in the presence of anti-LhbpA antibodies to demonstrate the surface expression of LhbpA by iron-limited leptospires. Organisms, maintained under low iron conditions and subjected to a temperature shift for about 5 h at 37ºC, showed marked agglutination upon addition of anti-LhbpA antibodies. The pre-immune serum from the same rabbit showed no effect; high iron cells also did not show any agglutination.

LhbpA expression is up-regulated both by temperature and iron limitation, both conditions likely to be experienced by the invading pathogen. It is 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 encounter conditions of iron deprivation *in vivo.* In the recently published list of temperature-regulated proteins (Lo *et al*., 2006), LhbpA (encoded by LB191) does not find a place,

as it is probably regulated both by temperature and iron. Earlier, Cullen *et al.* (2002), in their analysis of outer membrane proteins under different environmental conditions could not detect LipL36 and pL50 in leptospires grown under conditions of iron limitation and at temperatures above 30°C. The authors have indicated these organisms may not express these proteins during mammalian infection, citing additional evidence (Barnett *et al*., 1999) that LipL36 is down regulated *in vivo*. They also report that pL24 was expressed when more free iron was available. Another major OMP studied in detail by 2D gel electrophoresis by these authors is LipL32 and its cleavage products. Cleavage of LipL32 occurred when outer membranes were isolated from high iron organisms and not from low iron organisms, an observation that could be of biological significance as LipL32 is thought to play a role in hemolysis (Cullen *et al*., 2002).

All leptospiral serovars do not express LhbpA: PCR analysis have show the presence of *lhbpA* in the pathogenic serovars of *L. interrogans* whereas others including *L. borgpetersenii* serovar Tarassovi, *L. kirschneri* serovar Grippotyphosa and the non-pathogenic *L. biflexa* serovar Patoc and *L. meyeri* serovar Ranarum did not elaborate the *lhbpA* gene. Southern blot analysis of the chromosomal DNA identified the *lhbpA* gene in *L. interrogans* serovar Lai, Pomona and Autumnalis. No hybridisation was seen with DNA from the non-pathogenic serovar Patoc and the pathogenic serovars Tarassovi and Grippotyphosa. Whether the expression of LhbpA by some serovars is associated with the adaptation of these serovars to particular animal hosts remains to be answered. The absence of *lhbpA* gene in non-pathogenic

serovars together with its presence in the commonly found serovars of *L. interrogans* makes it a potential drug target and vaccine candidate.

Using random insertional mutagenesis (Louvel *et al*. 2005) and comparative and functional genomic analyses of iron transport and regulation in *Leptospira* spp (Louvel *et al*., 2006), this group have extensively studied iron transport in the saprophytic *L. biflexa* and extended their study to the pathogenic *L. interrogans*. They showed that *Leptospira* spp. were able to utilize exogenous siderophores. 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*. They also refer to the obvious question as to why saprophytes should have the ability to use hemin and hemoglobin as a source of iron. Though they identified *L. biflexa* mutants with *Himar1* insertion into a *fecA*-like gene, sequence analysis revealed that the N-terminal extension of FecA, which is involved in the regulation of the *fec* transport genes in *E. coli* (Braun *et al*., 1998 & Bagos *et al*., 2004), was not found in *Leptospira*. In addition, there was no evidence of *fecBCDE* homologues, suggesting that *Leptospira* must have an alternative pathway for ferric dicitrate transport. They also identified a TonB-independent receptor for iron acquisition, called FeoB, thought to transport Fe^{2+} in *E. coli* and other Gram-negative bacteria. Despite the identification of potential iron acquisition genes in *L. interrogans*, including LA2641 (similar to FhuA of *E. coli*), LA3149 (similar to heme receptors HmuR, HemR, and HutA of *Yersinia pestis*, *Y. enterocolitica* and *Vibrio cholerae*) and heme oxygenase (LB186),

no evidences have been provided to prove that they are in fact genes involved in iron acquisition.

The expression of iron-regulated proteins has been related to increased virulence in *V. cholerae*, *H. ducreyi*, *N. meningitidis* and *S. pneumoniae* (Henderson & Payne, 1994; Al-Tawfiq *et al*., 1999; Stojiljkovic *et al*., 1995 and Tai *et al*., 1993). Sequence alignment and phylogenetic analysis of LhbpA with other bacterial proteins showed that it shares evolutionary relationship not only with other hemin-binding proteins but also with other bacterial iron-regulated virulence proteins, especially the Irg proteins. This indicates that heme uptake system of the pathogens may help in the colonization of host tissues under low-iron conditions. The role of LhbpA, a heme utilization protein, in bacterial survival and its association with virulence determinants such as hemolysin is unknown. The studies done on *V. fluvialis* (Ahn *et al*., 2005) and *P. gingivalis* (Shibata *et al*., 2003) demonstrated that a heme uptake protein - deficient mutants of these pathogens showed severe reductions in virulence properties such as hemolysis and hemagglutination and their results suggest that enhanced hemolytic activity of the wild-type strain under iron-limited conditions liberates hemin from intracellular hemoglobin and makes it available for growth; hemolytic activity thus plays a beneficial role in bacterial iron acquisition in the host. Virulent strains of *Leptospira* have been shown to exhibit chemotaxis towards hemoglobin (Yuri *et al*., 1993)**.**

It is well known that iron regulates the expression of several bacterial virulence determinants (Salyers and Whitt, 1994). Pathogenic *Leptospira* produce hemolysins and sphingomyelinases, the latter 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.

Leptospiral hemolysins, mainly the sphingomyelinases form the second half of our study. First, we analysed the hemolysins from the genome data; subsequently, cloning and expression studies was done to show the iron-regulated expression and finally the clinical significance of the leptospiral sphingomyelinases was demonstrated by the identification of antibodies against these virulence determinants in the serum of patients with confirmed leptospirosis.

From the list of hemolysins and sphinogmyelinases in the *Leptospira* genome, sequence alignment and phylogenetic analysis was performed with the four sphingomyelinase (Sph) proteins, Sph1, Sph2, Sph3 and SphH (LA1027, LA1029, LA4004 and LA3540 respectively). When compared with other bacterial hemolysins, the Sph of *Leptospira* made a separate rooted branch in the phylogenetic tree and showed close evolutionary relationship with Sph of *Vibrio* and *Pseudomonas*. Though

the Sph proteins of *Leptospira* were different from other bacterial hemolysins, they shared high homology among themselves; evolutionarily Sph1 was more related to Sph2 whereas Sph3 was more related to SphH. Using ClustalW program, the region with the highest homology between *sph*1, *sph*2, *sph*3 and *sph*H was chosen and primers were designed to PCR amplify a 638 bp region (sph_{638}) from *Leptospira*.

The sph_{638} DNA fragment was PCR amplified from *L. borgpetersenii* serovar Ballum, cloned and expressed in pET 28a (+). Significant expression of $rSph_{638}$ was evident from the formation of inclusion bodies. Antibodies were generated against $rSph_{638}$ in rabbits. Our attempts to demonstrate the expression of these toxin molecules in the culture filtrate were unsuccessful. As our objective was to understand if iron levels controlled the expression of the sphingomyelinases, RT-PCR was used as a tool to demonstrate it's iron-regulated expression. The organisms, grown under high and low iron conditions were harvested and the RNA was used as the template in RT-PCR. The RNA template was ensured to be free of DNA template (by PCR amplification of the RNA template) and equal concentrations were used in RT-PCR. From the Results, it is evident that the $rSph_{638}$ transcript was significantly expressed under conditions of low limitation. The faint band in high iron cells could be due to the amplification of one or more of the sphingomyelinase genes that are not regulated by iron levels as the primers can anneal to all the four sphingomyelinases Though we identified the Fur box sequence upstream to *sph*1, *sph*2 and *sph*H, experimental evidence is required to prove which of these Fur boxes are functional. The Fur box (5'- GATAATCATAAT AATTTTG-3') located upstream of the *lhbpA* gene showed greater homology with the

consensus Fur box sequence of *E. coli* (5'- GATAATGATAATCATTATC -3') as compared to the Fur box upstream of the *sph* genes

Using specific primer in PCR amplification of the genomic DNA, most of the serovars in this study showed the 638 bp amplicon with the exception of *L. borgpetersenii* serovar Tarassovi strain perepelicin, *L. santarosai* Sarmin serovar Weaveri strain CZ-390, *L. noguchi*i serovar Louisiana strain LSU-1945 and *L. meyeri* serovar Ranarum strain ICF. Additional testing by hybridisation must be done to confirm the absence, if any of the gene(s). Other reports (Segers *et al*., 1992) show that the gene encoding hemolysins and sphingomyelinases are found in most of the serovars, though it is accepted that the latter are expressed only by the pathogenic serovars. The genome of the saprophyte *L. biflexa* also contains several genes encoding putative hemolysins (Louvel *et al*., 2006), with at least five genes that encode products that exhibit similarities to hemolysins as compared to the eight genes in *L. interrogans*. In addition, a putative hemolysin secretion system similar to the *E. coli* -hemolysin (HlyA) secretion system (Hornung *et al*., 1996 & Braun *et al*., 1993) was identified in *L. biflexa* (LEPBIa0357-LEPBIa0360), but no orthologous system was found in *L. interrogans*. The putative *L. biflexa* hemolysin secretion system comprises HlyB-, HlyD- and TolC- related proteins. The authors imply that *L. interrogans* could use alternative transport system.

This study, an important step in the understanding of iron acquisition and the role of this essential micronutrient on the virulence of pathogenic *Leptospira* has provided significant insights into leptospiral pathogenesis. Further studies are required

to understand the role of LhbpA in virulence and if pathogenic leptospires elaborate alternate mechanisms of iron acquisition. It is also important to analyse the inter-relationship between iron, the role of the outer membrane protein LipL32 and hemolysis. Molecular approaches and generation of mutants will enable a better understanding of iron acquisition in both pathogenic and saprophytic leptospires.

Conclusions

In conclusion, we have obtained an insight into the iron acquisition system in *Leptospira* and the role of iron on the expression of sphingomyelinases, know virulence determinants in the pathogenic leptospires. This is the first report on the identification of an iron-regulated, hemin-binding protein; we identified, cloned and expressed this TonB-dependant outer membrane protein that we named as LhbpA (leptospiral hemin-binding protein A). We have provided both *in silico* and experimental evidences to prove it is a hemin-binding protein. Agglutination of leptospires upon addition of anti-LhbpA antibodies indicates it to be a potential immunogen and thus a vaccine candidate. In addition, we observed that the *lhbpA* gene was found in *L. interrogans* serovars, thus making it a possible candidate for species-specific identification.

The saprophytic *L. biflexa* produces siderophores as assayed by the Chrome Azurol assay. Though we identified some iron-regulated proteins, a detailed analysis is required for a better comparison between the saprophytic and the pathogenic leptospires.

In addition, we studied the leptospiral hemolysins, specifically the sphingomyelinases. The sph_{638}, a common DNA fragment among the 4 shingomyelinase *sph1*, *sph2*, *sph3* and *sphH* was cloned and expressed. The iron-regulated expression of sph_{638}, shown by RT-PCR highlights the role of iron in virulence. Further studies are needed in this direction, as hemorrhage is commonly seen in the severe form of this disease. In clinical samples, antibodies against the

$rSph_{638}$ emphasises the *in vivo* relevance of these virulence determinants. The role of LhbpA and Sph-like hemolysin proteins in iron acquisition will help us to further understand the mechanism of pathogenesis of this organism. Future studies need to be done in experimental animal models to assess if LhbpA has a role in virulence. Also, as more than one mechanism of iron uptake is commonly seen in bacterial systems, other modes of iron acquisition need to be studied.

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1. Sritharan M and **Asuthkar S**. 2004. Iron-regulated proteins (IRPs) of *Leptospira biflexa* serovar Patoc strain Patoc I. *Indian Journal of Medical Microbiology*. 22(2): 92-96.

2. Sritharan M**,** Ramadevi S, Pasupala N, **Asuthkar S**, Tajne S. 2005 *In silico* identification and modeling of a putative iron-regulated TonB-dependant outer membrane receptor protein from the genome of *Leptospira interrogans* serovar Lai. *Online Journal of Bioinformatics*. 6 (2): 74-90.

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7. **Asuthkar S**, Velineni S and Sritharan M. Studies on the leptospiral sphingomyelinases (under preparation).