Hemin-binding proteins in pathogenic *Leptospira*: diagnostic potential of HbpA and the identification of second hemin-binding protein HbpB in *Leptospira interrogans* serovar Manilae

A thesis

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By

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CERTIFICATE

This is to certify that Ms. Subha Sivakolundu has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. Ordinance of this University. We recommend her thesis entitled "**Hemin-binding proteins in pathogenic *Leptospira*: diagnostic potential of HbpA and the identification of second hemin-binding protein HbpB in *Leptospira interrogans* serovar Manilae**" for submission for the award of the degree of Doctor of Philosophy in Animal Sciences of this University.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Hemin-binding proteins in pathogenic *Leptospira*: diagnostic potential of HbpA and the identification of second hemin-binding protein HbpB in *Leptospira interrogans* serovar Manilae**" is the result of the investigation carried out by me in the Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad and has not been submitted to any other University for the award of any degree or diploma.

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List of abbrevations

BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
bp	Base pair
EDTA	Ethylene diamio tetra - acetic acid
EDDA	Ethylene diamino N – N’ diacetic acid
EMJH	Ellinghausen – McCullough – Johnson - Harris
For	Forward
g	Gram
h	Hour
HAT	hypoxanthine-aminopterin-thymidine
HT	hypoxanthine and thymidine
Ig	Immunoglobulin
Kb	kilobase pair
kDa	kilodalton
MAb	Monoclonal antibody
mg	milligram
mL	Milliliter
mM	millimolar
MAT	Microscopic agglutination test
OMP	Outer membrane protein
o/n	Overnight
PAGE	Polyacrylamide gel electrophporesis
PEG	Polyethylene glycol
Rev	Reverse
SD	Standard deviation
TCGM	Tissue culture growth media
µM	micromolar

Abstract

Leptospirosis is an emerging zoonotic disease that is of concern globally and in India. The spirochetal pathogen belonging to the genus *Leptospira* causes high morbidity and mortality in animals and humans. Due to favoured transmission in monsoon seasons, this disease often breaks out as epidemics seasonally. The disease is usually self-limiting in humans but can progress to the severe fatal form known as Weil's disease.

The disease is usually self-limiting and the majority of patients recover without any adverse effects. The symptoms associated with the disease include severe body pain, headache, fever that is often mistaken for the common flu. If left untreated, the disease can progress to Weil's disease, in which damage is seen in different organs, particularly liver, kidney and the lungs. Hemorrhagic manifestations and tissue damage is often fatal. Proper and timely diagnosis is the major lacuna in the control of this disease and hence it is often under-reported as it is clinically similar to flu, dengue and malaria. The lack of rapid, economic and easy-to-perform tests are lacking for the diagnosis of leptospirosis. The serological microscopic agglutination test (MAT) is used as the 'gold standard'; however, due to several disadvantages it cannot be performed in routine diagnostic laboratories. Hence, there is a need to develop a simple serological test for the detection of antibodies against leptospiral-specific antigenic determinants. Understanding host-pathogen interactions result in the identification of novel molecules that can be used as diagnostic and vaccine candidates.

Acquisition of iron by pathogenic *Leptospira* spp. was first reported by Sritharan and her group who demonstrated direct acquisition of iron by pathogenic *L. interrogans* serovar Lai by the hemin-binding protein HbpA, an 81 kDa TonB-dependant outer membrane protein up-regulated upon iron limitation. HbpA is present in pathogenic serovars and is absent in saprophytic leptospires. This, coupled to the *in vivo* expression of the protein made HbpA an ideal candidate for diagnosis. PCR amplification of *hbpA* from the genomic DNA of several clinical isolates identified all serovars belonging to *L. interrogans* spp.

As the focus of this study was to develop an ELISA-based test for the serodiagnosis of leptospirosis, the diagnostic potential of HbpA was evaluated in

both bovine and human serum samples by ELISA. Serum samples from patients with systemic leptospirosis were screened by MAT, HbpA-ELISA and the commercial PanBio ELISA. The performance of HbpA-ELISA was superior to PanBio ELISA and it was observed that ELISA-based screening identified larger number of samples compared to MAT. The latter is a specific test for leptospirosis but, due to its serovar specificity can give false negative if the respective serovar was no included in the reference panel for screening. This was reflected strongly in patients with leptospiral uveitis, 50% of whom tested MAT-negative, with the two ELISA tests identifying the majority of the cases with confirmed clinical symptoms of the disease. The mean anti-HbpA antibody titres in both the groups were significantly higher in patients when compared with controls (P<0.05) that included subjects with non-leptospiral uveitis, cataract and endemic healthy normal subjects. HbpA-ELISA showed higher sensitivity (93%) and specificity (58%) over PanBio ELISA.

Screening of bovine serum samples by HbpA-ELISA was compared with MAT and ELISA based LilL41 and LipL32, two potential diagnostic antigens, specific for pathogenic serovars. The test showed a κ value of 0.920 for HbpA-ELISA vs LipL41-ELISA and 0.887 for HpbA-ELISA vs LipL32-ELISA indicating good agreement of HbpA with the two leptospiral antigens. All the three antigen-based ELISA tests showed 100% sensitivity with MAT; the specificity, however was low (~ 63%), for reasons of not including a larger panel of serovars. Since no epidemiological data is available in India on the prevailing serovars in a specific geographical region, a random panel of serovars had to be chosen. Thus, HbpA-ELISA was effective in the testing of both human and bovine serum samples and can possibly replace the cumbersome MAT.

Early detection of the disease is of paramount importance in the development of control measures. The organisms, upon entry into the mammalian host remain in circulation for about a week. During this period, culture is possible but due to the fastidious nature of the pathogen, it has not been easy to culture from blood samples. Since antigen detection is more feasible, we developed monoclonal antibodies against HbpA. Four monoclones 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C, belonging to IgG2b sub-class expressed high titers of anti-HbpA antibodies, with negligible cross-reactivity with other bacterial pathogens. MAbs

7C2.1C and 10E3.1C were specific for the C-terminal region of HbpA protein as determined by western-blot analysis and indirect ELISA. These MAbs, when used as primary antibody in sandwich ELISA were able to detect 0.48 ng of rHbpA.

The essentiality of *hbpA* was studied in a *hbpA* mutant strain of *L. interrogans* serovar Manilae. The *hbpA* mutant strain M601, generated by random transposon mutagenesis was a kind gift by Adler and his group. In the presence of hemin as the sole source of iron, the mutant strain was unable to grow and showed no increase in cell number for almost 6-7 days after inoculation. Growth, though seen after 7 days was much lower compared to the wild-type (WT) strain. RNA transcripts of the two strains grown under high (10 ug Fe / mL), low (200 µm EDDA) and hemin (1 µg in medium pre-incubated with 200 µm EDDA) showed changes in the levels of several iron-regulated genes, previous reported by microarray analysis. In this study, an interesting observation was the six-fold increase in the level of LA3149, annotated as encoding a Ton-dependant outer membrane protein, possibly a hemin-receptor. Since this was indicative of a second hemin acquisition system, the gene was clones and expressed. The recombinant protein, proved to bind hemin-agarose beads was called as hemin-binding protein B (HbpB). Homology modeling with Phyre program using ferripyoverdine (Fpv) receptor from *Psedomonas aeruginosa* as template showed HbpB to fold into the characteristic β-barrel and the globular plug domain. The presence of the N-terminal TonB box, presence of the FRAP-NNHL motif with a conserved histidine residue located between the two motifs (aa 550) confirmed it was a TonB-dependant, hemin-binding protein.

In conclusion, the potential of HbpA as a diagnostic antigen and the identification of HbpB as a second hemin-binding protein in the absence of HbpA are the major achievements from this study.

CHAPTER I

REVIEW OF LITERATURE

1.1. Introduction

Leptospirosis, first described by Adolf Weil in 1886 is a zoonosis that occurs throughout the world, its incidence being highest in tropical regions. More than 500,000 cases of severe leptospirosis occur each year, with case fatality rates exceeding 10% (Abela-Ridder *et al.*, 2010).

The spectrum of disease caused by leptospiral infection is extremely wide and varies from sub-clinical inapparent to severe multi-organ syndrome involving liver, kidney and lungs either alone or in combination (Bharti *et al.*, 2003). Pulmonary hemorrhage is increasingly being recognized as a major complication in several outbreaks of leptospirosis in developing countries (Truong & Coburn, 2011). Leptospirosis however, can bes wrongly diagnosed as the symptoms overlap that of other diseases like common flu, dengue fever, hantavirus infection, encephalitis, viral hepatitis and malaria (Levett, 2001).

Patients with leptospirosis are treated with penicillin or doxycycline that is effective in killing the pathogen (Levett, 2001). However, early diagnosis is necessary since antibiotic therapy provides greatest benefit when initiated early in the course of illness. The difficulty in diagnosing leptospirosis clinically highlights the need for laboratory diagnosis. The fastidious nature of the pathogen and its structural features do not allow for the easy identification by culture and common staining techniques. Serological diagnosis is done by demonstration of anti-leptospiral antibodies by the conventional technique called Microscopic agglutination test (MAT). The serovar specificity, technical skill needed for interpretation of MAT and maintenance of live organisms do not render this technique user-friendly to any investigating laboratory or hospital. Disease diagnosis is currently done by ELISA and latex agglutination techniques using commercial kits. Endemicity of the disease however demands the identification of an antigen that will identify active infection.

1.2. Identification of the causative organism of leptospirosis

The clinical manifestations of the icteric form of human leptospirosis was first described by Adolf Weil in Heidelberg in 1886 (Weil, 1886). Since then, Weil's syndrome, characterized by splenomegaly / jaundice / nephritis is synonymous

with leptospirosis. In 1907, Stimson isolated leptospires from a patient believed to have died of yellow fever (Stimson, 1907). His subsequent research highlighted that the bacteria were concentrated in the renal tubules and were shaped like a question mark. This gave rise to the name *Spirocheta interrogans* (Stimson, 1907). The causative organism of leptospirosis, demonstrated independently in Japan was called *Spirochaeta icterohaemorrhagiae* (Inada & Ido, 1915) while in Germany it was termed as *Spirochaeta icterogenes* (Uhlenhuth & Formme, 1916). Both the groups isolated and cultivated pathogenic leptospires. Saprophytic *Leptospira* called *Spirochaeta biflexa* was identified in fresh water. Noguchi studied the microorganism and proposed a new genus *Leptospira* meaning 'thin spiral' in 1917 as he found the organism to be morphologically different from all known genera of spirochetes (Noguchi, 1918).

Leptospirosis in animals was identified as a separate clinical entity in 1850 about 30 years prior to the time Weil described the disease. In 1898, an epidemic in dogs was recorded in Stuttgart, Germany, but only 28 years later when the etiologic agents were discovered, it was realised that the disease in dogs and humans was caused by microorganisms of identical morphology (Beran, 1994). During the first half of the 20th century, there was evidence to show that morphologically and serologically identical leptospires could affect all known mammals and some lower vertebrates. By the late 1940s and early 1950s, leptospirosis in domestic animals had been established as a disease of major significance in veterinary medicine and public health (Beran, 1994).

1.3. Taxonomy and classification

Leptospires belong to the Division-Gracillicutes, Class-Scotobacteria, Order-Spirochaetales and Family-Leptospiraceae. Leptospiraceae has three genera viz., *Leptospira*, *Leptonema* and *Turneria*. Presently two different classification systems are being used; one is based on serological characterization and other on genetic relatedness.

1.3.1. Serological classification

Prior to 1989, the genus *Leptospira* was divided into two species based on antigenic relatedness - *L. interrogans*, comprising all pathogenic strains and *L. biflexa*, comprising saprophytic strains (Levett, 2001). Saprophytic strains differ

from pathogenic strains by growing at 13°C and in the presence of 8-azaguanine (225 µg / mL) as well as by forming spherical cells in 1 M NaCl.

Both *Leptospira* species were divided into numerous serovars, defined by agglutination after cross-absorption with homologous antigen. Serovars that are antigenically related have traditionally been grouped into serogroups (Kmety & Dikken, 1993). *L. interrogans* is comprised of more than 250 serovars arranged into 25 serogroups (Cerqueira & Picardeau, 2009) while *L. biflexa* consists of 65 serovars arranged in 38 serogroups (Kmety & Dikken, 1993). The system of serogroup nomenclature has no taxonomic standing, but is retained because presumptive serogroup determination by serologic testing has some epidemiologic value (Levett, 2004).

1.3.2. Genotypic classification

Leptospires are classified into a number of species defined by their degree of genetic relatedness, determined by DNA-DNA hybridization (Ramadass *et al.*, 1992). The term genomospecies is used to indicate a species determined on the basis of DNA relatedness (Yasuda *et al.*, 1987). There are currently 20 named species (Table 1.1) including pathogens, non-pathogenic saprophytes and species of indeterminate pathogenicity (Cerqueira & Picardeau, 2009; Varni *et al.*, 2013).

Table 1.1 Genotypic classification of *Leptospira* spp.

Class	Species
Pathogenic	*L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii* (Yasuda *et al.*, 1987)*, L. kirschneri, L. alexanderi* (Brenner *et al.*, 1999) *and L. alstonii* (Smythe *et al.*, 2013)
Non-pathogenic	*L. biflexa, L. meyeri, L. yanagawae* (Smythe *et al.*, 2013)*, L. vanthielii* (Smythe *et al.*, 2013)*, L. wolbachii, L. kmetyi, L. terpstrae* (Smythe *et al.*, 2013)
Indeterminate pathogenicity	*L. wolffii, L. licerasiae, L. inadai* (Yasuda *et al.*, 1987)*, L. fainei* (Perolat *et al.*, 1998)*, L. broomii*

Molecular approaches like multiple locus sequence typing (MLST) (Ahmed *et al.*, 2006), restriction endonuclease analysis (REA) (Savio *et al.*, 1994), pulsed field gel electrophoresis (PFGE) (Herrmann *et al.*, 1992), restriction fragment length polymorphism (RFLP) (Zuerner *et al.*, 1993), arbitrarily primed PCR (Perolat

et al., 1994), variable number of tandem repeats (VNTR) analysis (Majed *et al.*, 2005) and fluorescent-amplified fragment length polymorphism (FAFLP) (Vijayachari *et al.*, 2004a) have been used for the characterization of leptospires at the species and sub-species level.

The re-classification of leptospires on genotypic relatedness is taxonomically correct and provides a strong foundation for further understanding of these organisms using genomic data (Brenner *et al.*, 1999). However, the molecular classification is incompatible with the system of serological classification which has been useful for clinicians and epidemiologists. Moreover, there is a pressing need for simple molecular tools for identification of species and serovars (Levett, 2004).

1.4. Features of leptospires

1.4.1. Morphology

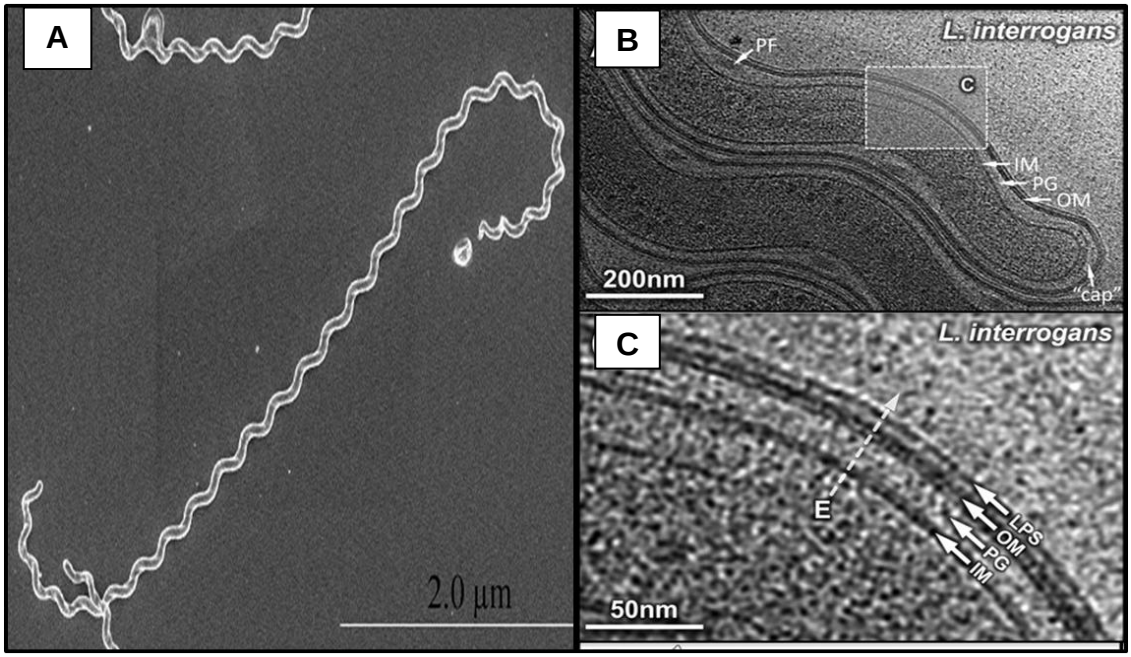
Leptospires are approximately 0.1 µm wide and 6-20 µm long with distinctive hooked ends (Fig. 1.1). They are too thin to be seen by staining with conventional staining techniques including aniline dyes but can be stained faintly by Giemsa stain. They are best visualized by dark-field microscopy due to their characteristic cork-screw movement. Leptospires are best stained by silver impregnation techniques (Faine *et al.*, 1999).

Leptospira has two periplasmic flagella, one attached sub-terminally at each end, that extend toward the cell's center without overlapping (Goldstein & Charon, 1988). Although the flagella lies inside the spirochete's outer membrane, they are integral to cell shape and motility (Faine *et al.*, 1999). In tissue and within phagocytes, organisms can assume a spherical or granular appearance (Faine *et al.*, 1964). Their narrow helical form allows them to burrow into tissue (Plank & Dean, 2000).

1.4.2. Culture characteristics

Leptospires are obligate aerobes with an optimum growth temperature of 28-30°C. They grow in simple media enriched with vitamins B_2 and B_{12}, long-chain fatty acids and ammonium salts. They cannot utilise glucose as a source of carbon

(Baseman & Cox, 1969) and depend on β-oxidation of fatty acids to meet their carbon and energy needs (Henneberry & Cox, 1970). Because of the inherent



(Stewart *et al.*, 2012) (Raddi *et al.*, 2012)

Fig. 1.1 Electron micrographs of *Leptospira*

Panel A shows the scanning electron micrograph of *L. biflexa* serovar Patoc strain Patoc I. Panel B represents the ultrastructure of intact *L. interrogans* reconstructed by cryo-electron tomography. The outer membrane (OM), inner membrane (IM), peptidoglycan layer (PG), and periplasmic flagellum (PF), the "cap" at the cell end, and a spherical body (SB) can be discerned from the picture. Panel C represents the zoom-in view revealing the structural details of the cell envelope of *L. interrogans.*

toxicity of free fatty acids, these must be supplied either bound to albumin or in non-toxic esterified form. Leptospires are commonly grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Ellinghausen & McCullough, 1965a, b) containing 1% bovine serum albumin and Tween 80 as source of long-chain fatty acids at pH 6.8-7.4. Rabbit serum containing high levels of vitamin B_{12} is required for their growth (Ellis & Michno, 1976) and serum containing media include Korthof's media (peptone, NaCl, $NaHCO_3$, KCl, $CaCl_2$, KH_2PO_4, Na_2HPO_4), Fletcher's media (peptone, beef extract, NaCl) and Stuart's medium (NaCl, Na_2HPO_4, NH_4Cl, $MgCl_2$, L-asparagine, KH_2PO_4, glycerol) (Faine *et al.*, 1999).

Liquid media is often used for growth and proliferation of leptospires while semi-solid medium containing 0.1-0.2% agar is used for long-term maintenance. In such semi-solid media, growth reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. This growth is related to the optimum oxygen tension and is known as a Dinger's ring or disk.

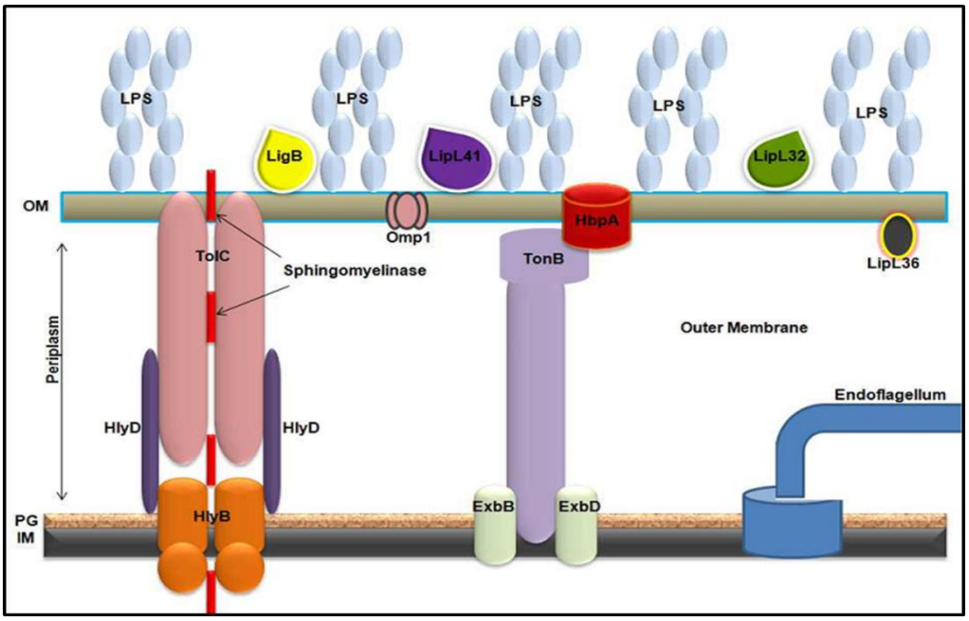
Growth of leptospires is often slow on primary isolation, and cultures have to be retained for about 13 weeks before being discarded. Growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil, gentamicin, nalidixic acid or rifampicin, that have no inhibition effect on leptospires (Faine *et al.*, 1999).

1.4.3. Leptospiral membrane architecture

Leptospira exhibits a surface architecture that resembles both Gram-negative and Gram-positive bacteria. Double membrane constitution separated by a periplasmic space supports Gram-negative bacteria whereas attachment of peptidoglycan to the inner membrane resembles Gram-positive nature (Fig. 1.2). Within the outer membrane, the lipopolysaccharide (LPS) constitutes the main antigen for *Leptospira*. LPS is composed of an endotoxic lipid A anchor, a conserved core oligosaccharide and an oligosaccharide or polysaccharide known as the O antigen. Leptospiral LPS is highly variable; its variations are thought to be the major antigenic determinant defining differences between approximately 230 serovars and contributing to serovar-specific immunity (Faine *et al.*, 1999). It is structurally and immunologically similar to LPS from Gram-negative organisms. Nevertheless, it is relatively non-toxic to cells or animals, being as much as 12 times less lethal for mice when compared with *E. coli* LPS (Faine *et al.*, 1999).

The two major types of outer membrane proteins (OMPs) are lipoproteins and transmembrane proteins. Lipoproteins become associated with membranes via a hydrophobic interaction between the N-terminal lipid moiety and the lipid bilayer phospholipids (Cullen *et al.*, 2004; Haake & Matsunaga, 2010). Lipoproteins can be localized to one or more of four cellular compartments: the periplasmic leaflet of the inner membrane, the periplasmic or outer leaflets of the outer membrane, or external to the outer membrane (Pinne & Haake, 2009).

LipL32 is the most abundant lipoprotein and accounts for 75% of the outer membrane proteome. This lipoprotein is highly conserved among pathogenic *Leptospira* spp. and is expressed in leptospires during acute lethal infections (Haake *et al.*, 2000). It is also known as hemolysis-associated protein-1 (HAP-1) as it is linked with hemolysis and has the ability to bind Ca^{2+}, laminin, collagen type IV and fibrinogen (Hauk *et al.*, 2009; Hoke *et al.*, 2008). Loa22 is the second most abundant outer membrane lipoprotein conserved in non-pathogenic and pathogenic *Leptospira*. The other important lipoproteins that have been well characterised include the outer membrane lipoproteins LipL41 (Haake *et al.*, 1999; Shang *et al.*, 1996), LipL21 (Cullen *et al.*, 2003), LipL46 (Matsunaga *et al.*, 2006), LigA (Palaniappan *et al.*, 2002), LigB (Lin & Chang, 2008), LenA (Verma *et al.*, 2010) and inner membrane lipoproteins such as LipL31 (Cullen *et al.*, 2005), LruA and LruB (Verma *et al.*, 2005).



(Sritharan, 2012)

Fig. 1.2 Schematic depiction of leptospiral membrane architecture

The inner membrane (IM) is closely associated with the peptidoglycan (PG) cell wall, which is overlaid by the outer membrane (OM). The leptospiral OM contains a mixture of LPS, surface-exposed lipoproteins (LipL32, LipL41, LigB) and transmembrane proteins (Omp1, HbpA). The hemin-binding protein HbpA is an example of a TonB-dependant outer membrane receptor that mediates the transport of hemin via the TonB protein located in the IM along with ExbB and ExbD. TolC, in association with HlyC and HlyD

forms a channel from the IM to the OM that possibly mediates the export of sphingomyelinase from the cytoplasm to the outside.

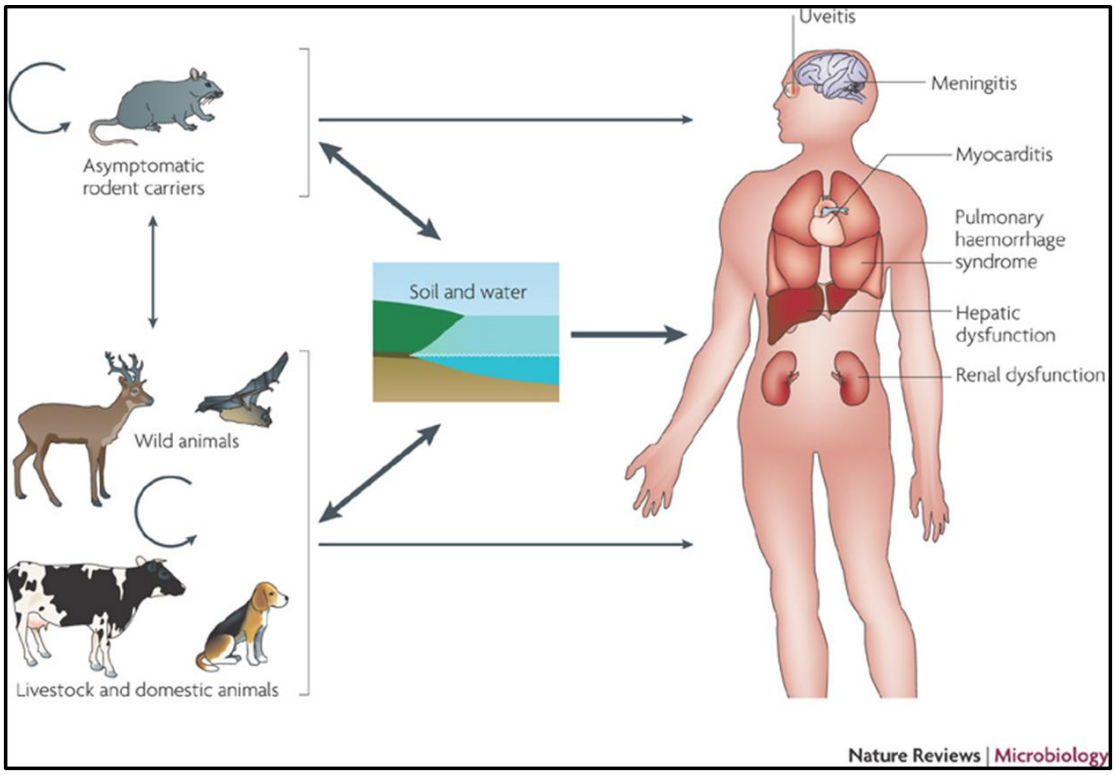
Transmembrane OMPs are typically integrated into the lipid bilayer by amphipathic β-sheets arranged in a barrel-like structure. The best described transmembrane OMP is the trimeric porin OmpL1 (Haake *et al.*, 1993; Shang *et al.*, 1995). Other important proteins include the TonB-dependant outer membrane proteins and the TolC proteins. The former, present on the outer membrane of Gram-negative organisms mediate the transport of several important nutrients like metal ions and vitamin B_{12}. TonB-dependant hemin-binding protein HbpA is an iron-regulated protein conserved in pathogenic *Leptospira* spp. and is involved in uptake of iron (discussed in detail in Section 1.14.) (Asuthkar *et al.*, 2007). The other transmembrane proteins that have been described include OmpL36, OmpL47, OmpL37, OmpL54 (Pinne & Haake, 2009), GspD (Haake & Matsunaga, 2010), FecA (Louvel *et al.*, 2005), OstA, Omp85, OmpA (Haake & Matsunaga, 2010) and TlyC (Carvalho *et al.*, 2009).

1.5. Modes of transmission

Leptospirosis is a zoonotic disease and affects both humans and animals. Wild and domestic animals serve as reservoir hosts with rodents playing a major role in disease transmission. Leptospirosis is maintained in nature by chronic infection of the renal tubules of maintenance hosts. These animals remain symptom-free and shed leptospires via urine into the surrounding environment. Transmission to other animals can be either direct or indirect. Direct transmission occurs when leptospires from tissues, body fluids or urine of acutely infected or asymptomatic carrier animals enter a new host and establish infection. Indirect transmission occurs when an animal or human acquires leptospirosis by exposure to environmental surface water or soil that is contaminated with their urine of carrier animals (Fig. 1.3). Leptospires can survive for long periods of time in the environment and probably multiply when the conditions are favourable. The usual portal of entry into the host is through abrasions or cuts in the skin or via the conjunctiva (Faine *et al.*, 1999).

Humans are accidental hosts and acquire infection due to several occupational and recreational or avocational exposures. Veterinarians, hunters,

animal handlers, miners and workers in livestock farming and slaughter house are at increased risk of exposure. Majority of cases are acquired either through occupational exposure to water, as in rice farming or other agricultural activities, flooding after heavy rains, or exposure to damp soil and water during recreational activities such as adventure tourism (Bharti *et al.*, 2003; Levett, 2001).



(Ko *et al.*, 2009)

Fig. 1.3 The cycle of leptospiral infection

Mammalian species excrete leptospiral pathogens in their urine and serve as reservoirs for their transmission. The pathogens are maintained in wild and domestic animals by transmission among rodent species. The disease is transmitted to humans by direct contact with reservoir animals or by exposure to environmental surface water or soil that is contaminated with their urine.

1.6. Epidemiology of leptospirosis

Leptospirosis is presumed to be the most widespread zoonosis in the world (Levett, 2001). The incidence of infection is much higher in tropical countries than in temperate regions (Fig. 1.4). This is due both to longer survival of leptospires in the environment in warm, humid conditions and to greater opportunities for human exposure. The incidence of leptospirosis is seasonal, peaking in summer or fall in

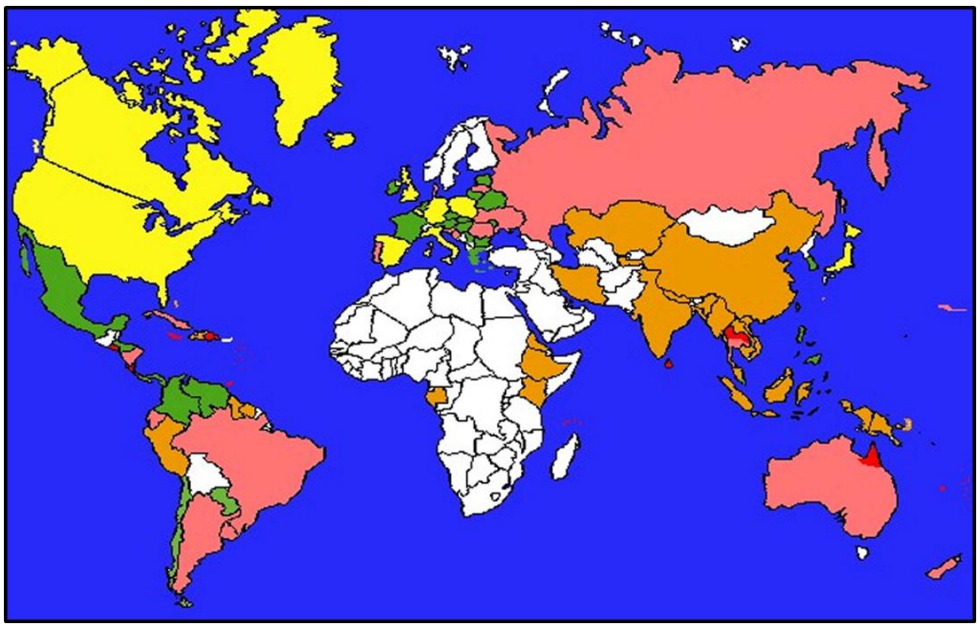
temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm climate regions, where rapid dessication would otherwise prevent survival of the organisms in the environment (Levett, 2004). WHO estimates the incidence of leptospirosis between 0.1-1 cases / 100000 population / year in temperate or non-endemic areas and between 10-100 cases / 100000 population / year in humid, tropical and endemic areas (WHO, 2012). An estimated 300,000-500,000 severe cases occur each year, with case-fatality reports of upto 30% (Hartskeerl, 2006).

1.6.1. The Indian Scenario

The history of leptospirosis in India dates back to the end of nineteenth century. During the period 1890-1930 there had been several reports of cases of disease presenting with acute fever, jaundice and haemorrhagic tendencies (Barker, 1926; Chowdry, 1903; Woolley, 1913). Many of these cases were suspected to be due to leptospirosis on clinical grounds (Barker, 1926). In 1929, the presence of leptospirosis in the Andamans was confirmed by isolating the organism from several patients with Weil's disease (Taylor & Goyle, 1931). It was the first report of bacteriologically confirmed leptospirosis from India. Since the 1980s, there have been increasing numbers of leptospiral cases reported in the states of Orissa (Jena *et al.*, 2004; Sehgal *et al.*, 2002), Maharashtra (Bharadwaj *et al.*, 2002; Karande *et al.*, 2003), Karnataka, Tamil Nadu (Chu *et al.*, 1998; Muthusethupathi *et al.*, 1995; Ratnam *et al.*, 1983, 1993), and Kerala (Kuriakose *et al.*, 1997).

Leptospirosis is endemic in the Andaman Islands. During the late 1980s, seasonal outbreaks of a mysterious febrile illness called the Andaman Haemorrhagic Fever (AHF) were reported. This was proved to be leptospirosis in 1993 (Sehgal *et al.*, 1995). 524 cases of AHF (leptospirosis) were reported from 1988-97. The disease presented as a febrile illness with pulmonary haemorrhage during post-monsoon periods. As the disease had a predominant pulmonary involvement, a leptospiral etiology was never considered (Sehgal *et al.*, 1995). In a study conducted in 2006, seroprevalence rate of leptospirosis was observed to be high in people engaged in activities such as agriculture, sewage cleaning, animal handling, animal slaughtering and forestry. The overall seroprevalence of 52.7% was reported in this high-risk population (Sharma *et al.*, 2006). A seropositivity rate

of 23.6% was reported among children in Andaman & Nicobar Islands (Vijayachari *et al.*, 2004b). At present, Andaman Islands has probably the highest incidence rates of leptospirosis in the country with figures ranging between 50-65 cases / 100,000 per year (Sehgal, 1998).



(Pappas *et al.*, 2008)

Fig. 1.4 Global incidence of human leptospirosis.

Colors reflect incidence, in declining order: red, pink, green, yellow. Gold reflects areas with probable, but not estimated, high incidence. White reflects absence of data.

Many places in South India are known to be endemic to leptospirosis. These include Chennai (Arumugam *et al.*, 2011; Muthusethupathi *et al.*, 1995; Ratnam *et al.*, 1983, 1993), Madurai (Chu *et al.*, 1998; Rathinam, 2002) and Salem (Natarajaseenivasan *et al.*, 2002) in the state of Tamil Nadu; Kottayam, Alleppey and Kozhikode (Kuriakose *et al.*, 1997) in the state of Kerala and some areas in the state of Karnataka. The state of Kerala is the worst affected with cases of leptospirosis presenting with hepato-renal involvement and myocarditis as a common complication. In Tamil Nadu, leptospirosis has been recognized as an important public health problem. It is a major cause of renal failure in Chennai (Muthusethupathi *et al.*, 1995). About 30% of pyrexia of unknown origin (PUO) cases in Chennai city during the monsoon period were found to have evidence of leptospiral infection (Ramakrishnan *et al.*, 2003). In 1994, an increase in the number of individuals with uveitis was noted at Aravind Eye Hospital, Madurai,

India, after an epidemic of leptospirosis in South India. The epidemic followed a severe flooding of Tamil Nadu district in the autumn of 1993 (Chu *et al.*, 1998; Rathinam, 2002). In the state of Andhra Pradesh, the disease remains largely under-reported. In 2007, a retrospective hospital-based study on human leptospirosis in Hyderabad region has been reported (Sritharan, 2012; Velineni *et al.*, 2007) in which, among 55 human sera tested by MAT, IgM ELISA and LeptoTek Dri-dot, *L. interrogans* serovar Lai (68%) emerged as a predominant serovar (Velineni *et al.*, 2007).

Mumbai is reported to be endemic to leptospirosis due to a heavy rainfall, poor sanitation and unhygienic conditions (Bharadwaj *et al.*, 2002). Following the Mumbai flood in July 2000, the city came to a standstill for two days. About two weeks later an outbreak of leptospirosis was reported in adults admitted to public hospitals. In a study conducted in 2003, 53 children of <12 years age admitted to a pediatrics department were tested positive for leptospirosis. Serological results indicated that almost one-third of the children had acute leptospirosis (Karande *et al.*, 2003).

In Orissa, following the super-cyclone that hit the coastal villages in the year 1999, nearly 19% of the studied subjects had febrile illness and serological evidence of leptospiral infection (Sehgal *et al.*, 2002). In 2013, a 10 year retrospective sero-epidemiological survey of leptospirosis was conducted in a tertiary care hospital in New Delhi. 26.9% of patients were found to be serologically positive with IgM ELISA. However, no difference was seen between sero-positive and sero-negative patients in clinical features and laboratory parameters. In *Leptospira* positive patients, co-infections with viral hepatitis E, malaria and dengue fever was observed (Chaudhry *et al.*, 2013).

1.6.2. World Scenario

In the Asia-Pacific region, countries with high incidence rates (> 10 per 100,000 population) are Bangladesh, Cambodia, Fiji, French Polynesia, Laos, Nepal, New Caledonia, Sri Lanka, Thailand and Vietnam. Countries like China, Malaysia, New Zealand, the Philippines and Mongolia have moderately high incidence rates (1-10 per 100,000 population) while low incidence rates (< 1 per 100,000 population) are seen in countries like Australia, Hong Kong, Japan, South Korea and Taiwan (Victoriano *et al.*, 2009).

The burden of human leptospirosis in Southeast Asia is huge. A recent study from the Thailand-Myanmar border confirmed leptospirosis as the second most frequent cause of fever in this region (Tangkanakul *et al.*, 2005). Taiwan exhibits a significant steady burden of disease which has emerged in the last 30 years (Yang *et al.*, 2005).

The Seychelles Islands showed the world's highest incidence (1000 per million population) with Icterohaemorrhagiae and Hurstbridge as the predominant serogroups (Yersin *et al.*, 1998). In the United States, the highest incidence was found in Hawaii with approximately 128 cases / 100,000 (Katz *et al.*, 2002). The disease is endemic in the Caribbean islands with more than 500 cases being confirmed leading to a cumulative annual incidence of >100 per million population (Pappas *et al.*, 2008).

Leptospirosis is the most common occupationally acquired infectious disease in New Zealand. The great majority of cases of leptospirosis occurs among livestock farm workers and meat processing workers (Crump *et al.*, 2001). In Europe, the disease is predominantly associated with occupational or recreational exposure. Mainland France has the highest prevalence in Europe with approximately 0.5 cases per 100,000 inhabitants; about 600 cases are diagnosed each year (Senior, 2010).

1.7. Clinical manifestation of leptospirosis

Leptospirosis has protean manifestations and mimics the clinical presentations of many other diseases. The disease is associated with a very broad spectrum of severity, ranging from subclinical illness followed by seroconversion to two clinically recognizable forms: a self-limited biphasic illness seen in approximately 90% of infections, and a severe, potentially fatal fulminant disease known as icteric leptospirosis or Weil's syndrome (Izurieta *et al.*, 2008; Levett, 2001).

1.7.1. Anicteric leptospirosis

Approximately 85-90% of leptospirosis cases are anicteric. Anicteric leptospirosis, is biphasic, with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (Izurieta *et al.*, 2008; Levett, 2001). The typical incubation period is 7-12 days (with a range of 2-20 days).

The acute septicemic phase of illness begins abruptly with a high remittent fever (38° to 40°C) and headache, chills, rigors and myalgias; conjunctival suffusion without purulent discharge; abdominal pain; anorexia, nausea and vomiting; diarrhoea; cough and pharyngitis and a pretibial maculopapular cutaneous eruption occurs rarely (Bharti *et al.*, 2003). Conjunctival suffusion (redness without exudate) and muscle tenderness, most notable in the calf and lumbar areas, are the most characteristic physical findings, but may occur in a minority of cases. Other less common signs include lymphadenopathy, splenomegaly and hepatomegaly. Leptospires can be recovered from blood and cerebrospinal fluid (CSF) and from urine, beginning about 5 to 7 days after the onset of symptoms (Levett, 2001).

Prior to the onset of the immune stage, there is a one to three day period with minimal symptoms. Symptoms then re-emerge in the immune stage which typically lasts 4-30 days (Izurieta *et al.*, 2008). The disappearance of leptospires from the blood and CSF coincides with the appearance of IgM antibodies. The organisms can be detected in almost all tissues and organs and in urine for several weeks, depending on the severity of the disease. In addition to the acute-phase symptoms described, the immune phase may be characterized by any or all of the following symptoms: jaundice, renal failure, cardiac arrhythmias, pulmonary symptoms, aseptic meningitis, conjunctival suffusion with or without hemorrhage, photophobia, eye pain, muscle tenderness, adenopathy and hepatosplenomegaly. In addition, aseptic meningitis, which is the hallmark of the immune stage, will usually occur and last a few days up to 1-2 weeks (Faine *et al.*, 1999; Feigin & Anderson, 1975).

1.7.2. Icteric leptospirosis

In contrast to anicteric leptospirosis, icteric leptospirosis or Weil's syndrome has much more severe manifestations. Between 5-10% of all patients with leptospirosis have the icteric form of the disease with a fatality rate of 5-15% (Levett, 2001). The prognostic signs include acute renal failure (oliguria, hyperkalemia, serum creatinine > 3.0 mg/dL), liver failure, hemorrhagic pneumonitis, respiratory insufficiency (dyspnea, pulmonary rales), hypotension and arrhythmias (Feigin & Anderson, 1975; Levett, 2001).

Jaundice occurs between the fourth and sixth day but may occur as early as the second day or as late as the ninth day. The liver is often enlarged and tender. Jaundice is due to hepatocellular necrosis, intrahepatic cholestasis and increased bilirubin load from absorption of tissue haemorrhage. Death rarely occurs due to hepatic failure (Bharti *et al.*, 2003; Levett, 2001).

Renal involvement is the most serious complication and is the most common cause of death in icteric leptospirosis (Muthusethupathi *et al.*, 1994). Tubulo-interstitial nephritis is the main cause of acute renal injury in leptospirosis (Yang *et al.*, 2001). Oliguria occurs in the second week but may occur as early as fourth day of illness. Renal manifestations range from urinary sediment changes (pyuria, albuminuria, hematuria and granular casts) to severe renal failure (Cetin *et al.*, 2004; Yang *et al.*, 2001). Renal complications are observed in all forms of leptospirosis regardless of severity of disease or of the infecting serogroup (Muthusethupathi *et al.*, 1994). Meningeal symptoms are also frequently observed but are overshadowed by hepatic and renal symptoms.

Severe pulmonary haemorrhagic leptospirosis (SPHL) has been recognized as an emerging clinical manifestation over the last two decades (Gouveia *et al.*, 2008; Truong & Coburn, 2011). SPHL carries a high mortality rate, and does not always coincide with the classic manifestations of severe leptospirosis (Weil's syndrome). On autopsy, patients with SPHL generally have haemorrhagic and / or necrotic lesions in numerous other sites (Chen *et al.*, 2007). The manifestations of SPHL reported by several different groups include dyspnea, crackles and sometimes massive hemoptysis (Truong & Coburn, 2011).

Most individuals recover from leptospirosis within 6-12 weeks after the onset of illness without further sequelae. However, full recovery may take years. Even with full recovery, there is no cross-protective immunity to other leptospiral serovars (Izurieta *et al.*, 2008).

1.7.3. Ocular manifestations

Uveitis is an important late complication that occurs around 2-6 months after systemic disease (Rathinam, 2002). The onset and severity of leptospiral uveitis are quite variable and does not correlate with the severity of systemic disease.

The incidence of ocular signs during acute systemic phase varies from 2% to 90%. Common ocular signs include unilateral or bilateral presentations, acute, non-granulomatous, anterior or pan-uveitis, hypopyon, optic disc oedema, retinal vasculitis and membranous vitreous opacities (Rathinam, 2002).

1.7.4. Leptospirosis in animals

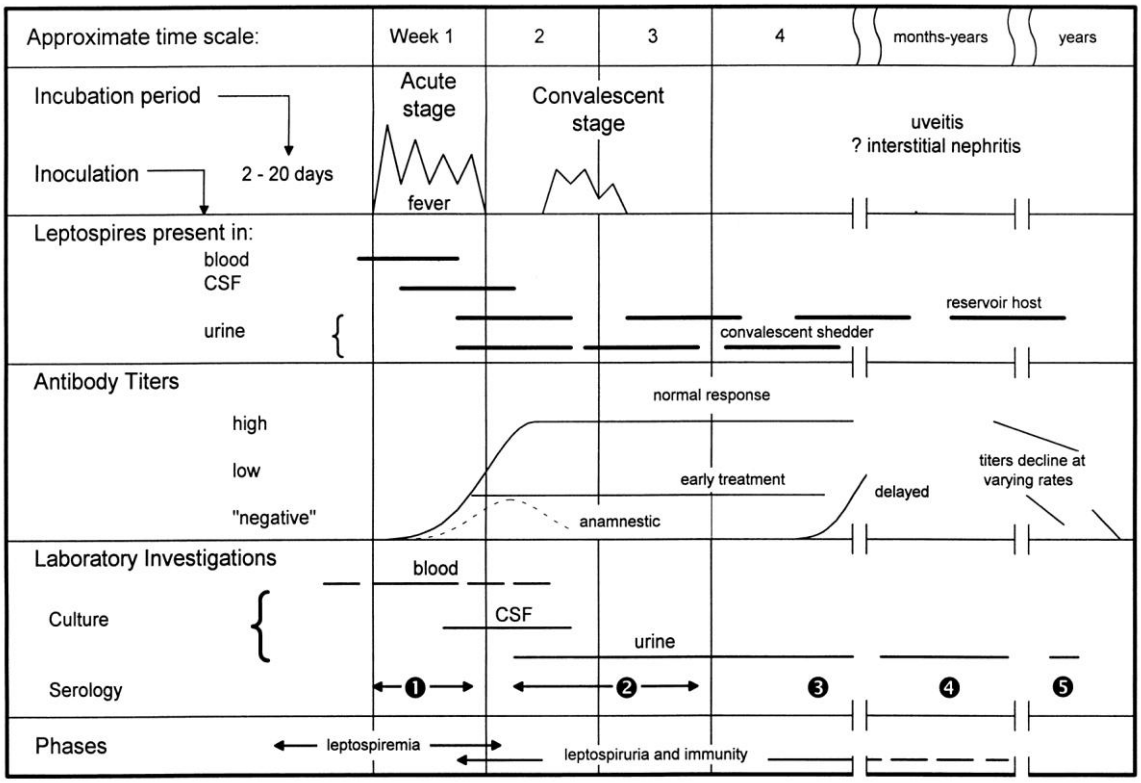
Leptospirosis in animals may range from mild to asymptomatic but severe and fatal illness also occurs. The symptoms occur 3-7 days after infection and are manifested by loss of appetite, irritability, fever, ruffled fur, red eyes, and sometimes diarrhoea. There may be signs of haemorrhages and jaundice. In milk-producing cattle, agalactia is generally observed. Congenital infection of foetuses in uterus may lead to abortion if the foetus dies. The aborted or stillborn foetus may be hemorrhagic or jaundiced or both and heavily loaded with leptospires (Adler *et al.*, 1994).

Animals that have recovered from acute leptospirosis may develop a carrier condition in which leptospires grow and may remain in the renal tubules for periods of days to years. Leptospires may also persist in other organs, notably the genital tract, the brain and the eye (Faine *et al.*, 1999). In horses especially, recurrent, autoimmune uveitis is well documented (Verma *et al.*, 2005).

1.8. Immune response of the mammalian host to leptospiral infection

The clinical presentation of leptospirosis is biphasic and thus necessitates the usage of appropriate diagnostic tests (Fig. 1.5). After infection, leptospires initially appear in the blood circulation and this stage is known as acute or septicemic or leptospiremic phase that lasts about a week. During this phase, diagnosis can be done using PCR or blood culture. This is followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine and is called the leptospiuric phase. In this phase, leptospires are cleared from the body by the host's immune response to the infection. However, they may settle in the convoluted tubules of the kidneys and be shed in the urine for a period of few weeks to several months. Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase. During this phase, diagnosis can be done using serological methods or urine culture.

Antibody production during the immune phase begins within 5-7 days after infection. Leptospires are cleared from the bloodstream and organs as the titres of serum agglutinating antibodies increase. The IgM antibodies usually appear before the IgG antibodies. These IgM titers may persist for months and possibly for years after the infection. The presence of human anti-leptospiral IgM sometimes persist



(Levett, 2001)

Fig. 1.5 Biphasic nature of leptospirosis and relevant investigations at different stages of disease

Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumptive infecting serogroup.

for several years. Concurrently, IgA begins to appear on approximately the fifth day of infection and may persist for as long as 9 months (Faine *et al.*, 1999; Levett, 2001).

The anti-leptospiral antibodies may be directed against genus-specific antigens that are shared by all leptospires or to antigens specific to a particular serovar or serogroup. LPS present in the outer-membrane is highly immunogenic

and is the primary target of humoral immunity. Passive immunization with polyclonal or monoclonal anti-LPS antibodies was able to confer protection to naive animals from leptospirosis (Jost *et al.*, 1986).

Patients with leptospirosis may produce antibodies that cross-react with several serovars during the initial phase of the disease. After the acute disease, cross-reactive antibodies gradually disappear as the immune response matures. Thus the genus-specific antibodies are detectable only for months while the serovar-specific antibodies persist for years. These serovar-specific antibodies are considered protective against reinfection with that serovar; however, they will not necessarily prevent infection with other serovars (Faine *et al.*, 1999).

Recent work has confirmed that immunity to leptospirosis is not limited to the humoral response. Mice require intact TLR2 (Chassin *et al.*, 2009) and TLR4 (Viriyakosol *et al.*, 2006) activation pathways to control a lethal infection. In contrast to immunity in hosts that are susceptible to acute leptospirosis, protective immunity against *L. borgpetersenii* serovar Hardjo in bovine reservoirs is cell-mediated. Cattle vaccinated with a killed *L. borgpetersenii* preparation when challenged with a virulent strain of serovar Hardjo resulted in INF-γ production and proliferation of both $CD4^+$ αβ and $WC1^+$ γδ T cells (Naiman *et al.*, 2001, 2002).

1.9. Diagnosis of leptospirosis

The diverse clinical presentations of leptospirosis necessitate definitive laboratory diagnosis. Though culture and direct demonstration of the pathogen is confirmative of the infection, culture from blood and biological specimens is not easy and is compounded by the difficulty in staining these organisms. Serological methods are the most preferred methods of detection due to the strong humoral immunity in this disease.

1.9.1. Culture confirmation

Leptospires can be isolated from blood, CSF and peritoneal dialysate during the leptospiraemic phase, before administration of antibiotics. A few drops of blood are inoculated into 5-10 mL of culture medium such as EMJH medium or modified Korthof medium supplemented with 5-fluorouracil and / or antibiotics to reduce the risk of contamination.

Urine can be cultured during the leptospiruria phase (~ 1 week after onset). The duration of urinary excretion varies, but may be several weeks (Bal *et al.*, 1994). Due to the acidic nature of human urine survival of leptospires is limited. Inoculation of urine into an appropriate culture medium should be done not more than 2 hours after voiding. Survival of leptospires in acidic urine may be increased by making it neutral.

Cultures are incubated at 28-30°C and examined weekly by dark-field microscopy, for up to 13 weeks (Levett, 2001).

1.9.2. Dark-field microscopy

Direct visualization of leptospires in blood or urine by dark-field microscopic examination has been used for diagnosis. However, artefacts are commonly mistaken for leptospires, and the method has both low sensitivity (40%) and specificity (61%) (Vijayachari *et al.*, 2001). A range of staining methods has been used to increase the sensitivity of direct examination including immunofluorescence, immunoperoxidase (carbazole, DAB) and Warthin-Starry silver staining. All of these suffer from the same drawbacks as dark-field microscopy: a high risk of false-positive and false-negative results (Levett, 2001).

1.9.3. Antigen detection

Detection of leptospiral antigens in clinical samples would offer greater specificity than dark-field microscopy while having the potential for greater sensitivity. A biotin / avidin double antibody sandwich ELISA developed using polyclonal IgG from rabbits immunised with *L. interrogans* serovar Hardjo type Hardjobovis could detect 10^4 leptospires of serovar Hardjo, but was less sensitive for other serovars (Champagne *et al.*, 1991). More recently, antigen detection in urine has been performed by dot blot ELISA using monoclonal antibody directed against the uncharacterized 35 kDa protein conserved in all pathogenic *Leptospira* spp. The assay detected leptospiral antigen in the urine of patients collected on their first day of hospitalization (Saengjaruk *et al.*, 2002). Gold nanoparticles coated with rabbit antibody specific to *L. interrogans* serovar Bratislava has been developed to detect leptospiral antigen in urine. The sensitivity of detection was 10 leptospires / mL with an assay time of 60 min (Chirathaworn *et al.*, 2011). Leptospiral antigens

were also detected in plasma in experimentally infected mice and guinea pigs using sandwich dot-ELISA with polyclonal and monoclonal antibodies (Sharma *et al.*, 2008).

1.9.4. Molecular methods

1.9.4.1. PCR assay

PCR detects DNA in blood within 5-10 days after the onset of the disease till 15th day with a bacterial load ranging from 10^5 to 10^9 leptospires / L (Musso & La Scola, 2013). Leptospiral DNA has also been amplified from urine (Bal *et al.*, 1994; Van Eys *et al.*, 1989), CSF (Merien *et al.*, 1995; Romero *et al.*, 1998), aqueous humor (Merien *et al.*, 1993) and tissues (Brown *et al.*, 2003).

Numerous targets for PCR detection of leptospires have been described, to amplify leptospiral DNA from either human (Merien *et al.*, 1995) or veterinary clinical material (Hernández-Rodríguez *et al.*, 2011; Talpada *et al.*, 2003), and of these, only two methods have been subject to extensive clinical evaluation (Gravekamp *et al.*, 1993; Merien *et al.*, 1992). Both methods were more sensitive than culture, but both these approaches have limitations. Gravekamp *et al.* (1993) reported two sets of primers (G1 / G2 and B64-I / B64-II) derived from genomic DNA libraries of serovar Icterohaemorrhagiae (RGA) and Bim (strain 1501). G1 / G2 primers do not amplify *L. kirschneri* serovars, necessitating the use of two primer pairs for detection of all pathogenic serovars. The primers described by Merien *et al.* (1992) amplify a 331 bp fragment of the *rrs* (16sRNA) gene of both pathogenic and non-pathogenic leptospires. Despite these shortcomings, these two primer pairs have been the most widely used for clinical studies (Brown *et al.*, 1995; Gumussoy *et al.*, 2009; Merien *et al.*, 2005; Talpada *et al.*, 2003). Several assays targeting genes such as those encoding *flaB* (Kawabata *et al.*, 2001), *lig* (Palaniappan *et al.*, 2005)*, lipl32* (Branger *et al.*, 2005b)*, hbpA* (Sridhar *et al.*, 2008)*, ompL1* (Reitstetter, 2006)*, rpoB* (La Scola *et al.*, 2006) *and* putative transcriptional regulator gene LA1137 (Liu *et al.*, 2006a) have been developed. A multiplex PCR assay to differentiate pathogenic and saprophytic leptospira has also been developed (Kositanont *et al.*, 2007).

1.9.4.2. Real-time PCR

Real-time PCR is faster than regular PCR and reduces the risk of false positive results by carry-over contamination. Several real-time PCR methods have been developed for the detection of pathogenic leptospires, but only a few have been clinically validated. Two of the assays target genes that are universally present in bacteria, the 16S rRNA (16S) gene (Smythe *et al.*, 2002) and the gene encoding DNA gyrase subunit B (*gyrB*) (Slack *et al.*, 2006). Other assays target genes such as *lipL32* (Levett *et al.*, 2005; Roczek *et al.*, 2008; Stoddard *et al.*, 2009), genomic locus LA0322 in *L. interrogans* serovar Lai (Merien *et al.*, 2005), *secY* (Ahmed *et al.*, 2009) and leptospiral immunoglobulin-like protein (*ligA* and *B*) (Palaniappan *et al.*, 2005) that are genes restricted to pathogenic species. The detection threshold is usually 10 to 100 leptospires / mL of blood or urine (Stoddard *et al.*, 2009). PCR-based diagnosis of leptospirosis however cannot identify the infecting serovar, which reduces its value in terms of epidemiologic research and public health. The real-time PCR instrument is also very expensive and may not be readily available in resource-poor laboratories.

1.9.4.3. Isothermal methods

In recent years, a growing number of isothermal amplification techniques have been developed for detecting pathogenic leptospires (Koizumi *et al.*, 2012; Lin *et al.*, 2009; Sonthayanon *et al.*, 2011). Unlike PCR, the LAMP (Loop-mediated isothermal amplification) method amplifies a target DNA sequence under isothermal conditions in approximately 1 h using DNA polymerase and six primers with high specificity and efficiency. The amplified DNA can then be detected visually for fluorescence or turbidity, without using electrophoresis gels. LAMP methods targeting genes *lipL41* or *rrs* have recently been developed for the quick detection of pathogenic leptospires (Koizumi *et al.*, 2012; Lin *et al.*, 2009; Sonthayanon *et al.*, 2011). The detection threshold ranges between 2 to 100 leptospires per reaction mixture (Koizumi *et al.*, 2012). The usefulness of LAMP for the diagnosis of leptospirosis should however be assessed in endemic zones.

1.9.5. Serological diagnosis

1.9.5.1. Microscopic agglutination test

Microscopic agglutination test (MAT) was first described by Martin and Pettit at the Pasteur Institute (Martin & Pettit, 1918). MAT is the reference gold-standard test for the serological diagnosis of leptospires because of its high sensitivity and specificity (Bharti *et al.*, 2003). MAT is a serogroup-specific assay based on the detection of the antibodies against leptospiral lipopolysaccharides. Live antigens representing different serogroups are incubated with serum samples and the agglutination is examined by dark-field microscopy. The end point is the highest dilution of serum that shows 50% agglutination when compared with control sample without any serum. Panels of live leptospires belonging to different serovars must be maintained in the laboratory which should include serovars representative of all serogroups and locally common serovars. The WHO recommends 19 serovars of 16 serogroups (WHO, 2003).

A serologically confirmed case of leptospirosis is defined by a four-fold rise in MAT titer to one or more serovars between acute-phase and convalescent serum specimens run in parallel. The cut-off value for a single serum depends on the seroprevalence of the disease in the area. In endemic areas of leptospirosis, a single titer of 400 or more in a symptomatic patient are generally accepted, but titers as high as 1600 or more have been recommended (Levett, 2001). A low titer equal to 100 in a febrile patient may indicate current infection in areas where leptospirosis is uncommon.

The interpretation of the MAT is complicated by cross-reaction between different serogroups, especially in acute-phase samples (Levett, 2001). Cross-reactivity in acute samples is attributable to IgM antibodies, which may persist for several years (Cumberland *et al.*, 2001). The major disadvantages of MAT are that it requires technical expertise, time consuming, laborious and maintenance of a panel of *Leptospira* cultures for which quality control must be employed periodically (Chappel *et al.*, 2004). The assay could also give false negative results especially when the strains that are used in the MAT panel do not match with those causing the disease. This often happens in the developing countries where prior knowledge of the prevalent pathogenic strains is often lacking due to resource-poor settings (Smythe *et al.*, 2009).

1.9.5.2. ELISA

Enzyme-based antibody assays were first described over 30 years ago (Adler *et al.*, 1980). Previously ELISAs were developed using disrupted whole leptospires or LPS as antigens which detected antibodies against both agglutinating LPS and subsurface non-agglutinating antigens, such as the heat shock proteins GroEL and DnaK. The antigens used for ELISA have been prepared using a variety of techniques, such as a hot phenol-water extraction (Thiermann & Garrett, 1983; Thiermann, 1983), formalin-extraction (Bourhy *et al.*, 2013), sonicated whole leptospires (Adler *et al.*, 1982; Cousins *et al.*, 1985; Fairbrother, 1985; Surujballi *et al.*, 1997a), acetic acid extraction (Surujballi *et al.*, 1997b), mechanical disruption (Trueba *et al.*, 1990), detergent extraction (Cho *et al.*, 1989; Goddard *et al.*, 1991), ethanol extraction (Fairbrother, 1985) or dodecyl sulphate extraction (Cho *et al.*, 1989; Goddard *et al.*, 1991; Yan *et al.*, 1999). Variations in the techniques used for preparation of antigens for the ELISA have contributed to the poor reproducibility of the results (Levett & Whittington, 1998). Development of a single specific antigenic reagent suitable for serological detection of infections with all serovars remains a great challenge.

Recombinant protein-based serological tests however offers great advantages as it does not require the maintenance of live organisms and antigen preparation is easier when compared with the tradition techniques. These assays can also attain high sensitivity and specificity because of the high concentration of antigens that can be used in these tests. Furthermore, the antigens are free of non-specific moieties, which are present in whole-cell preparations. Protein antigens that are conserved among pathogenic leptospires and are expressed only *in vivo* upon infection can be potential diagnostic markers. Recombinant protein-based assays for diagnosing leptospirosis have been developed, but the evaluation of these assays is controversial because of variations seen in the sensitivity and specificity of the assay performed (Table 1.2).

1.9.5.3. Commercial kits

Commercially available kits are generally whole *Leptospira*-based assays using non-pathogenic serovar Patoc as an antigen as it detects broadly cross-reactive antibodies. These assays are amenable for ‘point-of-care’ use and are easier to

Table 1.2 Recombinant protein-based ELISA for sero-diagnosis of leptospirosis

Antigen	Type	Source	Sensitivity		Specificity		Reference
			Acute cases	Conva-lescent cases	Patient control	Healthy control	
LipL32	IgG	Human	56	94	59	90	(Flannery *et al.*, 2001)
		Human	-	56	96		(Chalayon *et al.*, 2011)
		Canine	96		97		(Dey *et al.*, 2004)
		Bovine	100		100		(Bomfim *et al.*, 2005)
Omp1	IgG	Human	16	72	83	84	(Flannery *et al.*, 2001)
LipL41	IgG	Human	24	44	22	86	
		Human	-	30	97		(Chalayon *et al.*, 2011)
		Bovine	100		85		(Mariya *et al.*, 2006)
		Bovine	87		96		(Senthilkumar *et al.*, 2010)
Hsp58	IgG	Human	18	32	86	94	(Flannery *et al.*, 2001)
LipL21	IgG	Human	-	42	98		(Chalayon *et al.*, 2011)
Loa22	IgG	Human	-	58	100		
LigB	IgM	Human	81	-	93-100	90-97	(Croda *et al.*, 2007)
LigA	IgM	Human	92	-	98		(Srimanote *et al.*, 2008)
	IgG	Human	100	-	100		

perform but interpretation of results should be based on level of endemicity in the given region (Table 1.3). Field evaluations indicate that these assays have low sensitivities (39-72%) during acute-phase illness. The sensitivity however increases significantly between the first and second weeks of illness; testing of a late acute-phase sample (past 10^{th} day of illness) is therefore recommended (McBride *et al.*, 2005). Variability in test sensitivities was however observed between studies. These variations may be attributable to differences in the case definitions used, selection of the control population for test evaluation and prevalence of the different infecting serogroups in the serum of patient (Effler *et al.*, 2002).

Table 1.3 Evaluation trials of commercially available whole *Leptospira*-based serologic tests

Kit	Evaluation site	Sn[#]	Sp[$]	Reference[@]
PanBio Leptospira IgM, PanBio Pty Ltd. (Brisbane, Australia)	Kuala Lumpur	54	96	1
	Thailand	85	88	2
		90	55	3
	UK, Eire	90	94	4
	Hawaii	36	98	5
	Barbados	87	96	6
	Thailand, Palau, USA	90	97	7
	South Australia, Fiji	100	93	8
	Vientiane, Laos	60	65	9
Leptospirosis IHA test, MRL Diagnostics (Cypress, California, USA)	Barbados	92	94	6
	Thailand	73	97	2
	Hawaii	29	100	5
IgM dipstick assay, Integrated Diagnostics (Baltimore, USA)	UK and Eire	54	95	4
	Brazil	72-80	100	10
Leptospira Microcapsule (MC) agglutination test, Japan lyophilisation laboratory (Tokyo, Japan)	Thailand	78	95	2
Biolisa IgM ELISA, BIOS GmbH (Müenchen, Germany)	UK and Eire	100	85	4
Biognost IgM indirect fluorescent antibody (IFA) assay, Bios GmbH Labordiagnostik (Gräfelfing, Germany)	Hawaii	48	90	5
		40	85	
SERION IgM ELISA, Institut Virion, Serion GmbH (Würzburg, Germany)		48	98	
	South Gujarat, India	88-98	87	11
LEPTO Dipstick IgM test, Organon-Teknika Ltd. (Amsterdam, The Netherlands)	Hawaii	34	96	5
LEPTO IgM MICROLISA ELISA kit, J.Mitra & Co. Pvt. Ltd (India)	Madurai, India	60	65	12
Leptocheck dipstick assay, Zephyr Biomedicals (India)		80	59	
IgM lateral flow, KIT (Amsterdam, The Netherlands)	Hawaii, Indonesia, Netherlands, Seychelles	66	93	13

[#] Sensitivity (%); [$] Specificity (%)

[@]1 (Sekhar *et al.*, 2000); 2 (Naigowit *et al.*, 2000); 3 (Desakorn *et al.*, 2012); 4 (Zochowski *et al.*, 2001); 5 (Effler *et al.*, 2002); 6 (Levett & Branch, 2002); 7 (Bajani *et al.*, 2003); 8 (Winslow *et al.*, 1997); 9 (Blacksell *et al.*, 2006); 10 (Mcbride *et al.*, 2007); 11 (Panwala *et al.*, 2011); 12 (Rathinam *et al.*, 2012); 13 (Smits *et al.*, 2001)

1.9.5.4. Other serological techniques

Other serological tests have been developed: complement fixation (Pinto *et al.*, 1974), counter-current-immunoelectrophoresis (Myers, 1987), indirect hemagglutination (IHA) (Torten *et al.*, 1966), sensitized-erythrocyte-lysis (SEL) test (Tan, 1969), macroscopic slide agglutination, microcapsule agglutination (Arimitsu *et al.*, 1982) and Patoc slide agglutination (Andreescu, 1990). These tests are however rarely used as they lack specificity or sensitivity.

1.10. Prevention and control

Prevention of leptospirosis may be achieved by immunization, avoidance of high-risk exposures, adoption of protective measures and use of chemoprophylaxis, in varying combinations depending on environmental circumstances and the degree of human activity.

1.10.1. Preventive measures

Leptospirosis can be controlled by preventing transmission specifically from rodents and domestic animals. Avoidance of contact with contaminated or potentially contaminated bodies of water can decrease the risk of infection.

Personal hygiene and protective clothing are important preventative measures in high risk occupations. Gloves and face shields can help prevent infections when working with infected animals or tissues. Rubber boots can decrease the risk of infection in sewer workers and agricultural workers who may wade in urine-contaminated water.

1.10.2. Vaccines

Inactivated whole organisms, referred to as bacterins have been used as vaccines since the 1920s. They are prepared from whole leptospiral cells killed by a variety of methods like heat, formalin, phenol and irradiation (Faine *et al.*, 1999). Human vaccines containing killed leptospires have been used in China (Chen, 1985), Cuba (Martínez Sánchez *et al.*, 1998) and Russia (Ikoev *et al.*, 1999). However many of these bacterins were reported to be reactogenic for widespread human use and required repeated annual revaccination to retain immunity.

Vaccination of livestock can reduce urinary shedding and risk to human handlers. Commercial *Leptospira* vaccines are available globally for cattle, pigs

and dogs. Most bovine vaccines contain serovars Hardjo and Pomona; in North America, commercial vaccines also contain serovars Canicola, Grippotyphosa and Icterohaemorrhagiae (Faine *et al.*, 1999). Canine vaccines generally contain serovars Canicola and Icterohaemorrhagiae. But vaccination has proved to be only partially effective in animals due to the serovar restricted nature of vaccine induced immunity and the absence of serovars than those included in the vaccines (Adler *et al.*, 1994). Due to these limitations, efforts have focused on developing subunit vaccine using surface-exposed outer membrane proteins that are conserved among pathogenic serovars which can elicit a strong immune response.

The first evidence for the feasibility of this approach was the immunization of hamsters with *E. coli* membrane fractions containing recombinant LipL41 and OmpL1. This preparation induced significant protection against homologous challenge with *L. kirschneri* serovar Grippotyphosa (Haake *et al.*, 1999). LipL32 could induce a cross-protective immunity by DNA vaccine and adenovirus mediated vaccination but not with the recombinant protein (Branger *et al.*, 2001, 2005a). The most promising subunit vaccine candidates are the Lig proteins, which conferred a high-level protection in mice and hamster model of infection. The protection of Lig protein against heterologous challenge is yet to be determined as amino-acid sequence identity of Lig protein is 70-100% among the different *Leptospira* spp (Coutinho *et al.*, 2011; Palaniappan *et al.*, 2006).

1.10.3. Chemoprophylaxis

Antibiotic treatment is effective within 7 to 10 days of infection and it should be given immediately on diagnosis or suspicion. The antibiotic of choice is benzyl penicillin by injection in doses of five million units per day for five days (Faine *et al.*, 1999). Patients who are hypersensitive to penicillin may be given erythromycin 250 mg four times daily for five days. Doxycycline 100 mg twice daily for 10 days is also recommended. In less severe cases, oral antibiotics such as amoxicillin or ampicillin are generally prescribed. Third-generation cephalosporins and quinolone antibiotics also seem to be effective. A number of antibiotics, in particular dihydro-streptomycin has shown to be useful in the elimination of leptospires from carrier animals (Hartskeerl *et al.*, 2011).

The antibiotics could eliminate leptospires from accessible tissues but cannot reverse already established pathological changes, which must be treated by appropriate supportive therapy (Faine *et al.*, 1999).

1.11. Advances in leptospiral genetics

The availability of whole genome sequence information of *Leptospira* provides opportunities to understand the molecular basis for its pathogenesis. The emergence of new functional genomic technologies such as microarrays, in addition to recently developed targeted mutagenesis tools, creates new avenues to exploit the genome information in order to address the scientific imperatives for better diagnosis, vaccines and disease management.

1.11.1. Whole genome sequencing of pathogenic and non-pathogenic *Leptospira* spp.

The chromosome of *Leptospira* is characterized by a G + C content of 35-41 mol% and a genome size of 3.9-4.6 Mb depending on the strain and species (Adler *et al.*, 1994). There are currently nine published leptospiral genome sequences: two serovars (Lai and Copenhageni) of *L. interrogans* (Ren *et al.*, 2003), two strains of *L. borgpetersenii* serovar Hardjo (Bulach *et al.*, 2006), two strains of *L. biflexa* serovar Patoc (Picardeau *et al.*, 2008), two strains of *L. licerasiae* (Ricaldi *et al.*, 2012) and strain LT821 of *L. santarosai* serovar Shermani (Chou *et al.*, 2012). The genome is composed of two circular chromosomes. A third circular replicon, designated as p74, is found in *L. biflexa*, but not in pathogenic strains; however, 13 housekeeping genes found in p74 are present in the chromosome I of pathogenic strains.

1.11.2. Comparative genomics

Comparison of the genomes of the pathogens *L. interrogans*, *L. borgpetersenii*, intermediately pathogenic *L. licerasiae* and the saprophyte *L. biflexa* (Fig. 1.6) identified approximately 452 conserved pathogen-specific proteins and 1547 orthologs which represent the core genome of *Leptospira* (Ricaldi *et al.*, 2012). The core genome represented the functional categories that are involved in essential housekeeping functions.

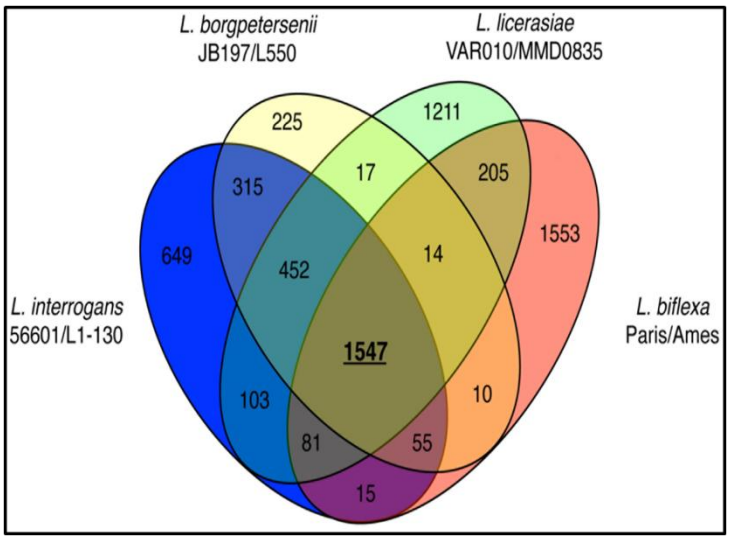
Comparison of genomes of pathogenic *Leptospira* spp. has yielded insights into the different transmission modes of these species. *L. interrogans* may be transmitted from carrier animals to other animals or humans either via direct or indirect mode. The indirect mode of transmission requires the organism to survive in the environment for extended periods of time until a mammalian host is encountered. *L. borgpetersenii,* however survives poorly in the environment and can be transmitted from host to host. This is evident from the genome of *L. borgpetersenii* being 16% smaller than genome of *L. interrogans*. The genes lost in *L. borgpetersenii* genome are those encoding proteins that impair adaptation to, and survival in, diverse environments, e.g., genes involved in environmental sensing and metabolite transport and utilisation. 12% of *L. borgpetersenii* genome encode transposases or are pseudogenes or gene fragments, as compared to 4% in *L. interrogans.* These factors impair the capacity of *L. borgpetersenii* to acquire nutrients and survive in environments external to a mammalian host (Bulach *et al.*, 2006).

One third of the *L. biflexa* genes are absent from the pathogenic species; these are mainly involved in environmental sensing and nutrient acquisition. Approximately 40% of the leptospiral genes encode proteins of unknown function. Out of the 655 proteins that are unique to *L. interrogans*, 78% have no known function. Likewise, 58% of the 308 unique *L. borgpetersenii* proteins have no defined function (Adler *et al.*, 2011). Thus, the pathogenic *Leptospira* spp. possess unique virulence determinants that cannot be identified by bioinformatics-based analysis involving comparison with other bacteria. Comparative genomic analysis needs to be combined with transcriptomic and mutagenic approaches to identify the determinants of virulence for this pathogen.

1.11.3. Whole genome microarray studies

Microarray technology has been useful in semi-quantitative or comparative analysis of genome-wide transcriptional patterns. This approach identifies gene regulation under different conditions, genes required for host adaptation and potential virulence factors in various bacterial species.

In order to identify leptospiral genes that are likely to be expressed during infection, microarray studies have been performed on leptospires grown at



(Ricaldi *et al.*, 2012)

Fig. 1.6 Comparative genomics of *Leptospira* spp.

Number of shared and unique genes amongst *L. interrogans*, *L. borgpetersenii*, *L. licerasiae* and *L. biflexa***.**

different temperatures (Lo *et al.*, 2009; Qin *et al.*, 2006), physiological osmolarity (Matsunaga *et al.*, 2007a), presence of serum (Patarakul *et al.*, 2010), iron-depleted medium (Lo *et al.*, 2010) and upon macrophage infection (Xue *et al.*, 2010).

Comparative analysis of datasets obtained from the microarray studies done in pathogenic *Leptospira* identified 14 genes (LA0594, LA0802, LA0816, LA1433, LA1456, LA1879, LA2014, LA2117, LA2778, LA3867, LA4299, LB102, LB186 and LB187) that were differentially expressed in conditions of osmolarity, iron limitation and temperature shift. Among them six genes (LA0802, LA1456, LA2117, LA3778, LA3867 and LB102), absent in *L. biflexa* could thus encode for potential virulence factors (Adler *et al.*, 2011). Heme oxygenase (LB186) and a putative permease (LB187) were up-regulated across three different experiments (physiological osmolarity, presence of serum and iron depletion), consistent with the importance of these genes in virulence and thus demonstrating the validity of microarray analysis in identifying potential virulence factors. Expression of *ligB* was up-regulated with temperature upshift to 37°C, high osmolarity and presence of serum, suggesting a role in virulence. A conserved hypothetical protein LA3867 was up-regulated with temperature upshift, high osmolarity and iron depletion. The other genes (LA0802, LA1456, LA2117, LB102) did not have a consistent

transcription pattern across the different conditions and it is possible that such genes are expressed at different stages of infection or in different host tissues (Adler *et al.*, 2011).

1.11.4. Mutagenesis studies: identification of genes required for leptospiral virulence

The genetic manipulation of leptospiral genome is still in an early phase in comparison with many other bacterial species. Previous attempts to perform classical genetic studies on *Leptospira* was prevented by the absence of naturally occurring plasmids in pathogenic leptospires and the slow growth of the bacteria in both solid and liquid media (Murray *et al.*, 2009a). With the discovery of the LE1 temperate leptophage replicating as a circular plasmid in *L. biflexa*, an *E. coli* - *L. biflexa* shuttle vector was developed, which contains LE1 origin of replication and antibiotic resistance markers (Girons *et al.*, 2000). This DNA can be introduced to leptospires by electroporation which replicates in *L. biflexa*, but not in pathogenic species (Girons *et al.*, 2000). The failure to transform pathogenic *Leptospira* could be due to competence, selective marker expression, recombination machinery, and / or DNA restriction and modification systems that differ in pathogenic versus saprophytic strains (Bourhy *et al.*, 2005).

Site-directed mutagenesis through allelic exchange was successful in the inactivation of several genes, such as flagellin (*flaB*) (Picardeau *et al.*, 2001), *recA* (Tchamedeu Kameni *et al.*, 2002), tryptophan biosynthetic gene (*trpE*) (Bauby *et al.*, 2003), heme synthetase gene (*hemH*) (Guégan *et al.*, 2003) and methionine biosynthesis genes (*metY*, *metX* and *metW*) (Louvel & Picardeau, 2007) in *L. biflexa* using a suicide plasmid. The first evidence of site-directed homologous recombination in pathogenic *Leptospira* species is the generation of *ligB* mutant (Croda *et al.*, 2008).

Libraries of transposon insertion mutants have been generated in different strains of *Leptospira* using the Himar1 mariner element (Bourhy *et al.*, 2005; Murray *et al.*, 2009a) (Table 1.4). While an extensive library of mutants was generated in *L. biflexa*, the pathogenic leptospires remain much less easily transformable with Himar1 (Murray *et al.*, 2009a). The random transposon mutagenesis system resulted in the generation of 926 mutants in *L. interrogans* of

which 721 affected the protein-coding regions of 551 different genes (Murray *et al.*, 2009a). Some of these mutants were tested for virulence in animal models (Table 1.4). These mutants provide a valuable resource to investigate the role of the gene in pathogenesis of *L. interrogans*.

Table 1.4 Leptospiral mutants tested for virulence in animal models

Mutated gene	Function	Serovar	Animal model	Reference
Gene mutations not resulting in attenuation				
lipL41	Hemin-binding protein	Manilae, Pomona	Hamster	(King *et al.*, 2013)
ligB	adhesion to ECM	Copenhageni	Hamster, Rat	(Croda *et al.*, 2008)
lipL32		Manilae	Hamster, Rat	(Murray *et al.*, 2009b)
Gene mutations resulting in attenuation				
hemO	heme oxygenase	Manilae	Hamster	(Murray *et al.*, 2008)
fliY	flagella motor switch	Lai	Guinea pig	(Liao *et al.*, 2009)
flaA	flagellar filament sheath	Manilae	Hamster	(Lambert *et al.*, 2012)
katE	Catalase	Manilae, Pomona	Hamster	(Eshghi *et al.*, 2012)
loa22	OmpA family protein	Lai	Guinea pig, Hamster	(Ristow *et al.*, 2007)
clpB	Chaperone	Manilae, Copenhageni	Gerbil	(Lourdault *et al.*, 2011)
colA	Collagenase	Lai	Hamster	(Kassegne *et al.*, 2014)
LA1641	LPS synthesis	Manilae	Hamster	(Murray *et al.*, 2010)
Lman1408		Manilae	Hamster	

1.12. Virulence factors of pathogenic *Leptospira* spp.

The molecular mechanisms of the pathogenesis of leptospirosis is not very well understood. Several candidate virulence factors have been identified whose combinatorial effect enables the pathogenic *Leptospira* to penetrate host-tissue barriers during infection, avoid the immune system, and cause harmful toxic effects to the host in order to establish a successful infection.

1.12.1. Motility and chemotaxis

Motility is an important factor for leptospiral survival as the *L. interrogans* genomes of both serovars Copenhageni and Icterohaemorrhagiae contain at least 79 motility-associated genes and 11 genes encoding methyl-accepting chemotaxis proteins (MCPs) (Nascimento *et al.*, 2004a; Ren *et al.*, 2003). The periplasmic endoflagella involved in propeller movement of bacterium is an important factor in tissue penetration and motility through highly viscous fluids. Moreover, chemotaxis for haemoglobin has been demonstrated, which indicates that *Leptospira* may be attracted to abraded skin surfaces (Yuri *et al.*, 1993).

The pathogenic *L. interrogans* has two *flaA* genes encoding for an outer flagellar sheath protein. The *flaA1* mutant generated by transposon mutagenesis retained normal morphology and virulence in a hamster model of infection but had reduced motility. The *flaA2* mutant however lacked the distinctive hook-shaped ends and translational motility in liquid and semi-solid media was lost. The mutant also failed to cause disease in animal models of acute infection (Lambert *et al.*, 2012). On the other hand, *fliY* gene which encodes for flagellar motor switch protein upon mutation in *L. interrogans* exhibited attenuated rotative motion in both liquid and semi-solid media. The *fliY* mutant showed much lower levels of adhesion to murine macrophages and apoptosis-inducing ability, and its lethality in guinea pigs was also significantly decreased (Liao *et al.*, 2009). These studies suggest that endoflagellar rotation and consequent bacterial motility may play an important role in the pathogenesis of leptospiral infection.

1.12.2. Leptospiral adhesins

The adhesion of leptospires to host tissue components is an initial and essential step for infection and pathogenesis. Attachment to host cells and extracellular matrix (ECM) components is necessary for the ability of leptospires to penetrate,

disseminate and persist in mammalian host tissues. It has been demonstrated that *L. interrogans* binds to a variety of cell lines, including fibroblasts, monocytes / macrophages, endothelial cells and kidney epithelial cells grown *in vitro* (Breiner *et al.*, 2009).

Surface lipoproteins present at the interface have been implicated to play roles as adhesins and notable are the Leptospiral immunoglobulin-like proteins (Lig proteins). LigA, LigB and LigC are induced under high osmolarity conditions. Lig proteins have been shown to bind strongly to laminin, fibronectin (plasma) and collagen IV. The expression of Lig protein by the pathogen is associated with virulence as diminished levels of the protein is seen in non-pathogenic strains and in pathogenic leptospires that have lost virulence after extensive subculturing in an artificial medium (Choy *et al.*, 2007).

Besides Lig proteins, a 36 kDa fibronectin-binding protein is an important adhesion protein responsible for virulence in leptospires (Merien *et al.*, 2000). Other ECM-binding proteins include a 24 kDa laminin binding lipoprotein named Lsa24 / LfhA (Barbosa *et al.*, 2006; Verma *et al.*, 2006) and a surface exposed Lsa21 which binds to laminin, collagen IV and plasma fibronectin (Atzingen *et al.*, 2008).

1.12.3. Lipopolysaccharide (LPS)

Resistance of pathogenic *Leptospira* to complement-mediated killing is thought to be mediated by LPS (Isogai *et al.*, 1986). Alterations in LPS have been implicated in evasion of host-response (Bolin *et al.*, 1989). Although leptospiral LPS is chemically and morphologically similar to that from other Gram-negative bacteria it is less endotoxic and activates human macrophages through TLR2 instead of TLR4 (Werts *et al.*, 2001). This differential recognition is attributed to the unusual composition of the leptospiral Lipid A moiety, and could be a strategy that pathogenic *Leptospira* may use to avoid adequate activation of immune cells, contributing to the establishment of the disease in humans (Nahori *et al.*, 2005; Que-Gewirth *et al.*, 2004).

The *L. interrogans* genetic locus encoding the biosynthesis of LPS is very large, spanning approximately 100 kb. Genome-wide random transposon

mutagenesis studies in pathogenic *Leptospira* spp. showed that this genetic region had an unusually low frequency of transposon insertion, suggesting that most of the genes contained therein are essential for the viability of the organism (Murray *et al.*, 2009a). Two mutants of *L. interrogans* serovar Manilae generated by random transposon mutagenesis with changes in LPS were found to be avirulent in the hamster model of infection, demonstrating that leptospiral LPS is essential for virulence (Murray *et al.*, 2010).

1.12.4. Surface proteins of leptospires

Outer membrane proteins (OMP) play key roles in pathogenesis by acting as adhesins, targets for bactericidal antibodies, receptors for various host molecules and as porins (Pinne & Haake, 2009). Therefore, the elucidation of surface-exposed proteins is critical for the better understanding of mechanism of pathogenesis. Leptospires can survive outside as well as inside the host, and some of the outer membrane proteins have been found to be differentially regulated between *in vivo* and *in vitro* conditions. For example, under *in vivo* conditions lipoprotein LipL36 is downregulated (Barnett *et al.*, 1999) while LigA (Palaniappan *et al.*, 2002), Qlp42 (Nally *et al.*, 2001), LipL32 and Loa22 (Nally *et al.*, 2007) were upregulated. A significant increase in the expression of OMPs Loa22, LipL32 and LipL21 in *L. interrogans* serovar Copenhageni recovered from an acutely infected guinea pig tissue and a chronically infected rat tissue, thereby demonstrates the effect of host environment on OMP expression (Nally *et al.*, 2007). Proteomic studies with leptospires grown in culture conditions that mimic the host environment like temperature (Lo *et al.*, 2009), osmolarity (Matsunaga *et al.*, 2005) and / or iron deprivation (Eshghi *et al.*, 2009) have also shown to affect the production levels of a number of OMPs.

1.12.5. Hemolysins: sphingomyelinases and phospholipases

Leptospiral hemolysins are postulated to be involved in the cell lysis, tissue damage due to sphingomyelinase, phospholipase or pore-forming activities (Bernheimer & Bey, 1986; Lee *et al.*, 2002). Through their action on host cell membranes, leptospiral sphingomyelinases are potentially involved in various aspects of pathogenesis, including tissue invasion, endothelial damage, immune evasion and nutrient acquisition (Narayanavari *et al.*, 2012a).

The hemolytic activity associated with *Leptospira* was reported in 1956 (Alexander *et al.*, 1956) while the sphingomyelinase activity in *Leptospira* cultures was first detected in the 1960's (Kasarov, 1969). Hemolytic and sphingomyelinase activities was expressed from a single gene in *L. borgpetersenii* serovar Hardjo that was later designated *sphA* (del Real *et al.*, 1989; Segers *et al.*, 1990). The sphingomyelinase encoded by *sphA* shared significant similarity to those found in *S. aureus* and *B. subtilis* (Segers *et al.*, 1990). Multiple sphingomyelinase sequences were detected in pathogenic members of *Leptospira* by low stringency southern hybridization using *L. borgpetersenii sphA* as a probe (Segers *et al.*, 1990). *SphH*, one of the sphingomyelinase homologs in the genome of serovar Lai, was identified from a genomic library using *sphA* as the probe (Lee *et al.*, 2000). The protein showed 75% similarity to SphA. SphH showed pore forming activity and did not express sphingomyelinase or phospholipase activity (Lee *et al.*, 2000, 2002).

Genome sequencing studies revealed that pathogenic *Leptospira* possessed multiple hemolysin genes (Nascimento *et al.*, 2004b; Picardeau *et al.*, 2008; Ren *et al.*, 2003). These pathogens elaborate several sphingomyelinase genes, whose numbers vary among the leptospiral species; five in *L. interrogans* (Nascimento *et al.*, 2004b; Ren *et al.*, 2003), three in *L. borgpetersenii* (Bulach *et al.*, 2006) and seven in *L. santarosai* (Chou *et al.*, 2012).

The analysis of these proteins in serovar Lai revealed that they shared a common exo-endophosphatase domain (EEPD), with the exception of *sph4* (Narayanavari *et al.*, 2012a). Modelling and sequence comparison of amino acid residues with the two well-characterized sphingomyelinases in *Bacillus cereus* and *Listeria ivanovii* identified Sph2 to be a true sphingomyelinase (Narayanavari *et al.*, 2012a, b). It contains the Glu53 and His151 and His286 residues required for its catalytic activity (Narayanavari *et al.*, 2012b). Sph2, in the presence of Mg^{2+} catalysed the cleavage of sphingomyelin to ceramide and phosphorylcholine and caused lysis of sheep erythrocytes. This enzymatic and hemolytic activity explains the toxic effects of this molecule on host cell membranes that are rich in sphingomyelin (Narayanavari *et al.*, 2012b).

Sphingomyelinases are expressed *in vivo* as reported in human subjects (Carvalho *et al.*, 2010) and experimental animals (Artiushin *et al.*, 2004). Sph2 is up-regulated upon raising the osmolarity and temperature of the growth medium to physiological levels (Lo *et al.*, 2006; Matsunaga *et al.*, 2007b). Iron limitation in culture media also resulted in the secretion of a 42 kDa protein, identified as sphingomyelinase using specific antibodies (Velineni *et al.*, 2009). A recent report showed that infection of mammalian cells caused the up-regulation of several sphingomyelinases (Wang *et al.*, 2012).

1.13. Host pathogen interaction: Role of iron

1.13.1. Iron requirement and availability

Iron is the fourth most abundant element in the earth's crust and, after aluminium the second most abundant metal. It is an important micronutrient for all bacteria except *Borrelia burgdorferi* (Posey & Gherardini, 2000) and lactobacilli (Imbert & Blondeau, 1998). It is a co-factor for enzymes involved in redox reactions and several metabolic pathways including DNA synthesis (Sritharan, 2000). Under physiological conditions, i.e., in the presence of oxygen and neutral pH, it is not available due to the rapid oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) and the subsequent formation of insoluble hydroxides. The concentration of Fe^{3+} in mammalian biological fluids is 10^{-18} M that is too low to support the growth of bacteria (Litwin & Calderwood, 1993).

Free iron catalyses the conversion of hydrogen peroxide to free radicals via the Fenton reaction (Pierre & Fontecave, 1999)

$$H_2O_2 + Fe^{2+} \longrightarrow HO^{\bullet} + OH^{-} + Fe^{3+}$$

The free radicals can react with macromolecules such as nucleic acids (DNA, RNA), proteins and lipids resulting in substantial damage that could be harmful to the cell. To defend against these toxic effects, the host sequesters free iron in iron-binding proteins (such as ferritin, transferrin, lactoferrin) and in heme and hemoproteins. Sequestration and compartmentalization of iron further reduces free iron concentration to 10^{-24} M within the animal host, which is far below the minimal nutritional requirements of bacteria (Raymond *et al.*, 2003).

1.13.2. Bacterial adaptations to iron-limitation

Microorganisms have adapted to conditions of iron-limitation by the elaboration of novel iron acquisition machineries (Neilands, 1995; Wandersman & Stojiljkovic, 2000). These include siderophore-mediated uptake and the direct removal of the protein-bound iron by specific receptors from the host-iron containing molecules like transferrin, lactoferrin, hemin and haemoglobin.

1.13.2.1. Siderophore-mediated iron acquisition

Siderophores are low molecular weight (500-1000 Da) Fe^{3+}-specific iron chelating compounds with binding affinity constant K_s ranging from 10^{22} to 10^{50}. Siderophores can extract iron from iron binding proteins such as transferrin, lactoferrin and ferritin and transport it to specific siderophore receptor (Wandersman & Stojiljkovic, 2000). They are secreted by both Gram-positive and Gram-negative bacteria and was first identified in the fungus *Ustilago* (Neilands, 1952). Siderophores are synthesized and secreted under conditions of iron limitation (Neilands, 1995). The number of different siderophores produced by bacteria, yeast and fungi is more than 500. Although chemically diverse, many of the siderophores produced by pathogenic bacteria can be classified as catechols, hydroxamates and hydroxyacid (Wandersman & Delepelaire, 2004).

The prototype of a catechol siderophore is enterobactin, a cyclic compound made up of three molecules of 2,3-dihydroxybenzoylserine connected via ester linkages first identified in *Salmonella typhimurium* (Pollack & Neilands, 1970) and in *E. coli* (O'Brien & Gibson, 1970). Enterobactin is transported across the outer membrane of *E. coli* via the FepA receptor. An example of a hydroxamate siderophore is aerobactin, synthesized by certain *E. coli* and *Shigella* strains (Payne, 1988). Yersiniabactin, produced by *Yersinia* spp., contains two thiazoline rings as well as a phenolate group, all of which have the potential for coordinating iron (Chambers *et al.*, 1996). Many pathogens also use siderophores produced by other microorganisms, collectively termed xenosiderophores.

1.13.2.2. Direct uptake of iron from host proteins

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

1.13.2.2.1. Transferrin and lactoferrin receptors

Transferrin and lactoferrin constitute a family of iron-binding glycoproteins having a molecular weight of 80 kDa. They bind to two Fe^{3+} ions per molecule along with two counter ions, usually CO_3^{2-}.

Extraction of the iron atoms from Tf and Lf is occurs *via* the TbpB / TbpA (Transferrin binding protein) and LbpB / LbpA (Lactoferrin binding protein) proteins, respectively. The ability to use Tf and Lf directly may be particularly important in the non-siderophore producing organisms and has been best described in the members of the *Neisseriaceae* and *Pasteurellaceae* families (Schryvers & Stojiljkovic, 1999). The TbpA / LbpA proteins are integral outer membrane lipoproteins that are peripherally associated with the outer membrane via an N-terminal lipid moiety (Noinaj *et al.*, 2012). TbpA is a TonB-dependent receptor that binds the C-terminal lobe of transferrin, where it extracts iron through a distortion of the iron-binding site and transports it across the outer membrane. TbpA alone is sufficient for transferrin utilization, but the co-receptor TbpB enhances the efficiency of uptake by binding transferrin and increasing its concentration for subsequent utilization by TbpA (Schryvers & Stojiljkovic, 1999). Molecular modelling suggests that the Lbps are functionally similar to the Tbps. In contrast to TbpB, LbpB does not increase the efficiency of lactoferrin uptake (Noinaj *et al.*, 2013).

1.13.2.2.2. Hemin and haemoglobin receptors

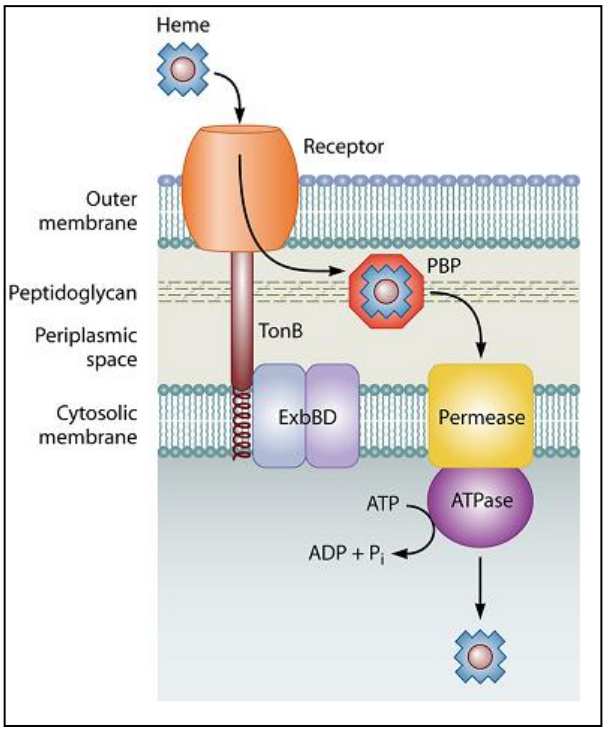
Approximately 70% of the iron in the human body is within heme (Runyen-Janecky, 2013). Heme uptake in Gram-negative bacteria involves a TonB-dependent outer membrane receptor, a periplasmic binding protein and an ABC transporter (Fig. 1.7). There are over 30 well-characterized outer membrane heme receptors that transport heme in Gram-negative pathogens, although there are many more putative receptors in genomic databases (Table 1.5). The hemin receptor is a membrane-spanning β-barrel protein with extracellular loops that bind

to free heme, host hemoproteins, or bacterial hemophores (Wilks & Burkhard, 2007). Most Gram-negative heme receptors share amino-acid homology, including conservation of FRAP / NPNL domains with a conserved histidine residue that coordinates with heme (Stojiljkovic *et al.*, 1995). Some heme receptors lack the conserved residues (e.g., PhuR from *Pseudomonas aeruginosa*) suggesting that there could be other motifs for heme coordination in outer membrane receptors (Tong & Guo, 2009).

Table 1.5 Microbial receptors for heme and heme-sequestering proteins

Organism	Gene	Substrate	Reference
Bordetella pertussis	*bhuR*	Heme, Hb	(Vanderpool & Armstrong, 2001)
Corynebacterium diphtheriae	*hmuT*		(Drazek *et al.*, 2000)
E. coli	*chuA*		(Torres & Payne, 1997)
Pseudomonas aeruginosa	*phuR*		(Ochsner *et al.*, 2000)
Vibrio cholerae	*hutA, hutR*		(Datta & Crosa, 2011; Litwin & Byrne, 1998)
Mycobacterium tuberculosis	*mmpL11*		(Tullius *et al.*, 2011)
Haemophilus influenzae	*hgpA, hgpB, hgpC*	Hb, hemopexin	(Morton *et al.*, 1999)
	hxuA	Heme, hemopexin	(Cope *et al.*, 1998)
Neisseria meningitidis	*hpuAB*	Hb, hemopexin	(Lewis *et al.*, 1998)
	hmbR	Heme, Hb	(Stojiljkovic *et al.*, 1996)
Porphyromonas gingivalis	*hmuR, HmuY*	Heme, Hb	(Liu *et al.*, 2006b; Simpson *et al.*, 2000)
	hbp35	Heme	(Shoji *et al.*, 2010)
Helicobacter pylori	*frpB1*	Heme, Hb	(Carrizo-Chávez *et al.*, 2012)
	frpB2	Hb	(González-López & Olivares-Trejo, 2009)

The energy for heme transport is provided by the electrochemical charge gradient of the cytosolic membrane and is delivered by the energy-transducing TonB-ExbB-ExbD protein complex. All heme outer membrane receptors have a conserved hydrophobic seven-amino acid motif 'TonB box' at the N-terminus through which the receptor interacts with TonB protein (Genco & Dixon, 2001).



(Parrow *et al.*, 2013)

Fig. 1.7 Bacterial heme acquisition in Gram-negative bacteria.
In Gram-negative bacteria, heme binds an outer membrane receptor that transports it to the periplasmic space by using energy transduced from the cytosolic membrane via the TonB / ExbBD complex. A periplasmic binding protein (PBP) transfers the heme to a membrane-spanning permease, and transport across the cell membrane is mediated by an ATPase.

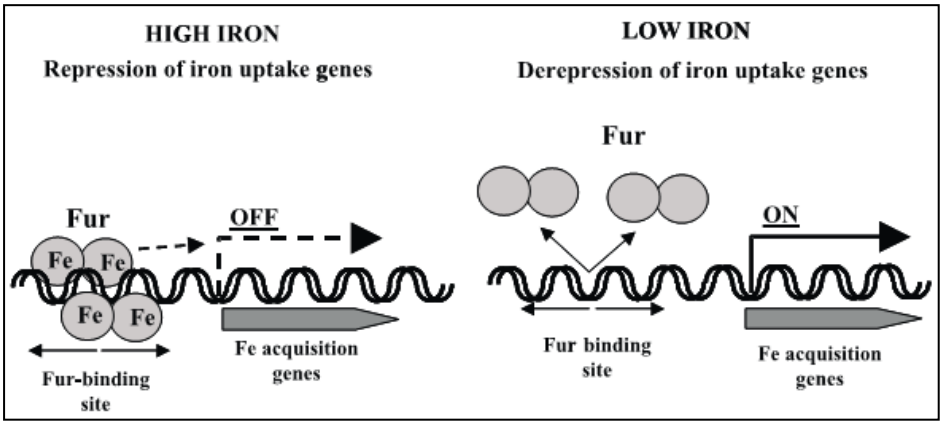
Once the heme molecule has been transported through the outer membrane receptor, ABC transport systems transport heme though the periplasm, inner membrane and into the cytoplasm. Each ABC transport system consists of a high-affinity periplasmic ligand-binding protein which shuttles heme through the periplasm and the two subunits of a cytoplasmic membrane permease. A peripheral membrane ATPase supplies the energy for transport (Runyen-Janecky, 2013). Upon entry into the bacterial cytosol, heme can be directly incorporated into bacterial hemoproteins, or degraded by bacterial heme oxygenases to release the iron (Andrews *et al.*, 2003).

1.13.3. Regulation of iron transport systems

Most genes encoding components of iron acquisition systems are not transcribed under iron-replete conditions because it would be energetically wasteful and

excess of iron uptake is cytotoxic to cell (Andrews *et al.*, 2003). One of the most common mechanisms of regulation of iron acquisition system is the expression of iron-responsive transcriptional regulators that repress transcription of iron acquisition genes when iron is plentiful. Iron-responsive transcriptional regulation by ferric uptake regulator (Fur) of *E. coli* is one of the most well studied regulatory mechanisim. Fur is a 17 kDa iron-binding protein that, when complexed with Fe^{2+}, blocks transcription by binding to a conserved operator sequence termed the Fur box within the promoter region of iron-regulated genes. In the absence of iron, Fur cannot bind to the Fur box, and repression is relieved (Fig. 1.8). The consensus Fur box in *E. coli* is a 19 base pair palindrome formed by two, nine base pair (bp) inverted repeats, separated by one bp: (GATAATGAT)A(ATCATTATC). Alternatively, this sequence can be defined as a series of 6 bp repeats.

In most bacteria where iron is required for growth, Fur or a similar protein acts as an important regulator of iron acquisition genes. In some bacteria where Fur is absent, iron acquisition is carried out by a similar metallo-regulator protein. In Gram-positive mycobacteria, DtxR (first identified in *Corynebacterium* spp.) and its homolog, IdeR, controls iron uptake respectively (Carpenter *et al.*, 2009).



(Andrews *et al.*, 2003)

Fig. 1.8 Schematic representation of Fur-mediated gene repression and activation

1.13.4. Iron-mediated regulation of virulence

Iron is one of the environmental signal that regulates the expression of genes required for survival and virulence in the host. Genes under negative control of iron are up-regulated in response to the low-iron levels in the host. These not only include iron-acquisition systems but also several toxins such as diphtheria toxin,

shiga toxin, *Pseudomonas aeruginosa* A toxin and numerous hemolysins (Litwin & Calderwood, 1993).

A classic example of iron-regulated expression of virulence factors is the diphtheria toxin from *Corynebacterium diptheriae*, which is under control of the DtxR regulator. The DtxR repressor, as the DtxR-Fe complex binds to the –10 region upstream of transcription start site of the *tox* gene encoding diphtheria toxin and blocks its transcription. Under low iron conditions, the repressor no longer binds and toxin production is increased. The expression of Shiga-like toxin (SLT-1), which interferes with eukaryotic ribosomal function, is repressed by Fur in enterohemorrhagic *E. coli* strains (Litwin & Calderwood, 1993). These examples indicate that the shift to low iron and hemin is frequently an indicator of host invasion to pathogens and results in coordinate control of factors required for virulence.

1.14. Iron and *Leptospira*

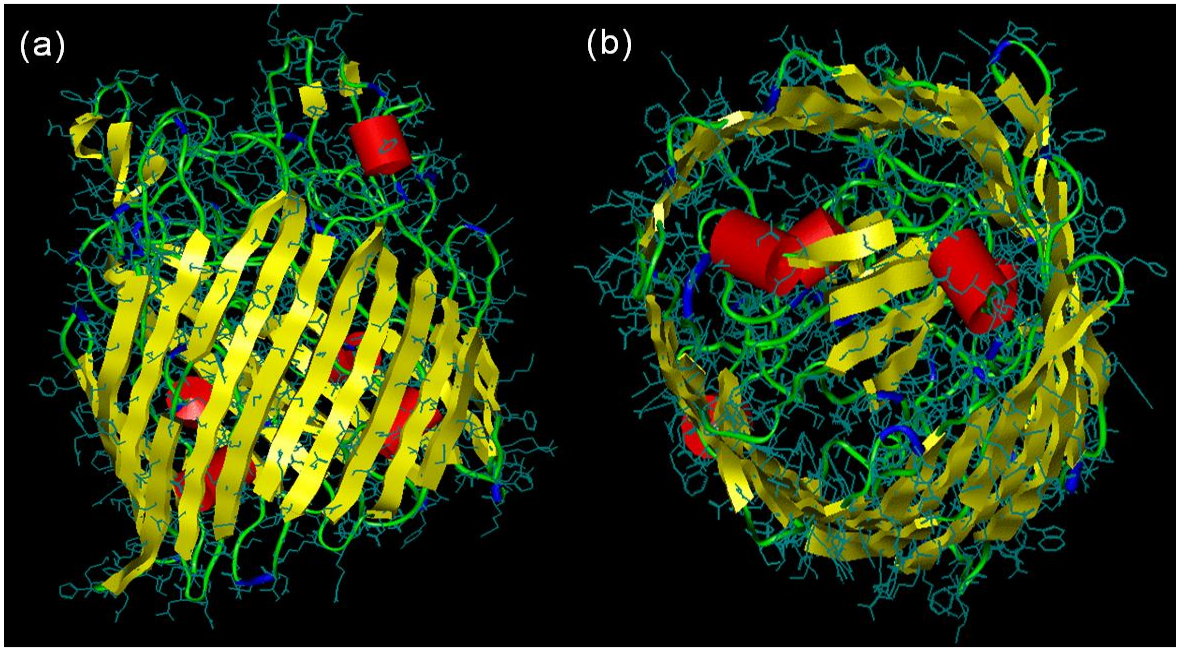
Iron is essential for the growth of both saprophytic and pathogenic *Leptospira* spp. (Faine, 1959). Cullen *et al.* (2002), in their study on outer membrane proteins showed the influence of temperature and iron on the expression of outer membrane proteins. Under low-iron conditions LipL36, LA0412 (pL24) and LA3469 (pL50) were downregulated. Processing of LipL32, the major outer membrane protein of pathogenic *Leptospira* spp., was shown to be affected by iron availability, possibly due to iron-regulated proteases (Cullen *et al.*, 2002). Genome analysis has revealed that *Leptospira* spp. possesses a complete set of genes for *de novo* synthesis of heme and is therefore capable of heme biosynthesis (Guégan *et al.*, 2003). Using random transposon mutagenesis with the saprophytic *L. biflexa*, Louvel *et al.* (2005) identified five hemin-requiring mutants; three of these had insertions in a gene encoding a protein with homology to FecA, the TonB-dependent ferric citrate receptor in *E. coli*, while the remaining two mutants had insertions in a FeoB-like protein, the product of which is required for ferrous iron uptake in many bacterial organisms.

Whole genome analysis of several *Leptospira* spp. revealed that the genes encoding the biosynthetic machinery for siderophore synthesis or secretion is absent (Ren *et al.*, 2003) and studies from our lab did not identify siderophores

(Asuthkar *et al.*, 2007). The first report on direct iron acquisition in pathogenic *L. interrogans* serovar Lai was from our lab (Asuthkar *et al.*, 2007; Sritharan *et al.*, 2005). Using an *in silico* approach, a putative TonB-dependent outer membrane receptor protein, called hemin-binding protein A (HbpA, LB191) was identified in the genome of *L. interrogans* serovar Lai by homology modeling using ferric-enterobactin receptor FepA of *E. coli* as the template (Sritharan *et al.*, 2005). This protein, modelled with the Insight II software showed a fold similar to ferric-siderophore receptors FepA, FhuA and FecA of *E. coli* (Fig. 1.9). HbpA has a characteristic β-barrel domain, plug domain and the N-terminal TonB box seen in TonB-dependant outer membrane receptor proteins (Sritharan *et al.*, 2005). The FRA / PP-NPNL motif associated with hemin-binding is present in HbpA. The full-length HbpA was cloned and the hemin-binding ability of HbpA was shown experimentally by assaying the inherent heme-dependent peroxidase activity of the bound hemin and by spectrofluorimetry (Asuthkar *et al.*, 2007). Immunofluorescence studies by confocal microscopy and the microscopic agglutination test demonstrated the surface localization of HbpA in *L. interrogans* (Asuthkar *et al.*, 2007). HbpA expression is upregulated by iron limitation and rise in temperature (Asuthkar *et al.*, 2007), which are conditions encountered by the pathogens within the mammalian host.

However it is not clear if the entire hemin molecule is internalized or the iron is released at the cell surface. The former probably occurs as *Leptospira* spp. possesses a heme oxygenase, encoded by *hemO*, present upstream of *hbpA* than can degrade the tetrapyrrole ring of the heme molecule, thereby releasing ferrous iron (Sritharan, 2012).

PCR amplification of *hbpA* using gene-specific primers and Southern blotting analysis (Asuthkar *et al.*, 2007) showed the presence of the gene only in pathogenic species and not in non-pathogenic *L. biflexa*. PCR-based detection of the *hbpA* gene in clinical isolates obtained from different geographical regions demonstrated the conservation of *hbpA* amplicon in all the serovars belonging to *L. interrogans* species (Sridhar *et al.*, 2008). The study also established the *in vivo* expression of HbpA by demonstrating the presence of anti-HbpA antibodies in serum of patients with leptospirosis (Sridhar *et al.*, 2008).



(Sritharan *et al.*, 2005)

Fig. 1.9. Homology-modelled structure of hemin-binding protein of *L. interrogans* serovar Lai.

(a) β-barrel structure (b) top view showing plug domain

Comparative global proteome analyses on *L. interrogans* serovar Copenhageni grown under the absence of iron and presence of serum showed an altered expression of 65 proteins, including upregulation of the virulence factor Loa22 and 5 novel proteins with homology to virulence factors found in other pathogens (Eshghi *et al.*, 2009). Microarray analysis of *L. interrogans* serovar Manilae grown under iron-limiting conditions showed an upregulation of 43 genes while 49 genes were downregulated (discussed further in Chapter IV, Discussion). Genes encoding proteins with predicted involvement in inorganic ion transport and metabolism (including TonB-dependent proteins and outer membrane transport proteins) were over-represented in the upregulated list, while 54% of differentially expressed genes had no known function. There were 16 upregulated genes of unknown function which are absent in *L. biflexa* which could possibly encode iron-regulated virulence factors.

Iron also regulated the expression of virulence determinants in *Leptospira*. Our lab is the first to show the direct evidence that *Leptospira* express sphingomyelinase under iron-limitating conditions (Velineni *et al.*, 2009).

Leptospiral sphingomyelinase was secreted into the medium as outer membrane vesicles (OMVs). It was detected as an OMV-associated 42 kDa protein in iron-limited culture of *L. interrogans* serovar Lai and was absent in the corresponding non-pathogenic *L. biflexa* (Velineni *et al.*, 2009).

Objectives of the study

A. Evaluation of the diagnostic potential of the hemin-binding protein HbpA
    i. Screening of serum samples for anti-HbpA antibodies by HbpA-ELISA
    ii. Generation of monoclonal antibodies against HbpA of *L. interrogans* serovar Lai and evaluation of their potential in antigen detection in serum samples
B. Identification of a second hemin-binding protein HbpB (LA3149) in *L. interrogans* serovar Manilae

CHAPTER II

MATERIALS AND METHODS

2.1. Source of chemicals

Bacterial grade media components were purchased from HiMedia (Mumbai, India). Routine analytical reagents and solvents were purchased from Qualigens (Qualigens Fine Chemicals Pvt. Ltd., India). Ellinghausen-McCullough-Johnson-Harris (EMJH) medium base, EMJH enrichment and Noble agar were purchased from Becton Dickinson and Company (MD, USA). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethylenediamine-*N*,*N'*-diacetic acid (EDDA), acrylamide, *N*,*N'*-methylenebisacrylamide, HAT (hypoxanthine-aminopterin-thymidine) media supplement (50X), 8-Azaguanine, red blood cell (RBC) lysing buffer, HT (hypoxanthine and thymidine) media supplement (50X), Freund's adjuvants, BCIP / NBT liquid substrate system, 3, 3′-diaminobenzidine (DAB) and bicinchoninic acid (BCA) protein estimation kit were purchased from Sigma-Aldrich Company (MO, USA). Pierce™ Rapid ELISA mouse mAb isotyping kit, IPTG (isopropyl-beta-D-thiogalactopyranoside), DreamTaq DNA polymerase, T4 DNA ligase, restriction enzymes, DNA and protein molecular weight markers were purchased from Thermo Fisher Scientific Incorporation (MA, USA). Reaction clean up kit, QIAquick PCR purification kit, RNeasy mini kit were purchased from Qiagen (Limburg, Netherlands). Fetal bovine serum (FBS), L-glutamine, antibiotic-antimycotic solution (100X), Dulbecco's modified Eagle medium (DMEM), TURBO DNA-free™ kit, Trizol reagent, Power SYBR Green master mix and SuperScript II Reverse Transcriptase were purchased from Life technologies (CA, USA). Ni-NTA His-Bind resin was purchased from Merck (Darmstadt, Germany). PanBio Leptospira IgM ELISA kit was procured from Inverness Medical Innovation Pty. Ltd. (QLD, Australia).

2.2. Leptospiral strains

The leptospiral serovars used in the study are as listed in Table 2.1

Table 2.1 List of *Leptospira* used in the study

	Species	Serogroup	Serovar	Strain
I[@]	*L. interrogans*	Australis	Australis	Ballico
		Autumnalis	Bankinang	Bankinang I
		Autumnalis	Rachmati	Rachmat

		Canicola	Canicola	Hond Uttrecht IV
		Sejroe	Hardjo	Hardjoprajitno
		Hebdomadis	Hebdomadis	Hebdomadis
		Icterohaemorrhagiae	Lai	Lai
		Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
		Pomona	Pomona	Pomona
		Pyrogenes	Pyrogenes	Salinem
	L. borgpetersenii	Ballum	Ballum	MUS127
		Javanica	Poi	Poi
		Tarassovi	Tarassovi	Perepelicin
	L. kirschneri	Canicola	Galtoni	LT1014
		Cynopteri	Cynopteri	3522C
		Grippotyphosa	Grippotyphosa	CH31
		Grippotyphosa	Grippotyphosa	Moskva V
		Ratnapura	Ratnapura	Wumalasena
	L. noguchii	Louisiana	Louisiana	LSU-1945
		Panama	Panama	CZ-214K
		Grippotyphosa	Canalzonae	CZ188
	L. weillii	Celledoni	Celledoni	Celledoni
	L. wolbachii	Codice	Codice	CDC
	L. santarosai	Sarmin	Weaveri	CZ-390
	L. biflexa	Andamana	Andamana	CH11
	L. meyeri	Ranarum	Ranarum	ICF
II[#]	*L. biflexa*	Patoc	Patoc	Patoc 1 (Paris L418)
	L. interrogans	Icterohemorrhagiae	Lai	56601
III[$]	*L. biflexa*	Semaranga	Patoc	Patoc1
IV[*]	*L. borgpetersenii*		*Hardjo-bovis*	L550
	L. interrogans		Manilae	L495

Source of leptospiral strains: [@] Regional Medical Research Center, ICMR, Port Blair, Andaman and Nicobar Islands, WHO Collaborating Centre for Diagnosis, Research, Reference and Training in Leptospirosis, India; [#] Dr. Rudy Hartskeel, KIT Biomedical

Research, The Netherlands; $ Aravind Medical Research Foundation, Madurai; * Prof. Ben Adler, Monash University, Australia.

2.3. Preparation of culture media for growth *of Leptospira*

2.3.1. Ellinghausen-McCullough-Johnson-Harris (EMJH) enrichment medium

0.23 g of EMJH base was dissolved in 90 mL of DDW in a 250 mL flask and autoclaved at 15 lbs / inch^2 pressure for 20 min. After cooling, 10 mL of enrichment was added aseptically, mixed well and stored at 4°C for future use. EMJH semi-solid medium was prepared by adding 0.15 g of Noble agar to EMJH base before autoclaving.

2.3.2. EMJH-BSA medium for growing leptospires under iron-regulated conditions

All glassware were made iron free by soaking in 2% methanolic KOH overnight followed by an overnight soaking in 8 N HNO_3 and subsequent washings with glass DDW.

0.23 g of EMJH base was dissolved in 75 mL of pre-autoclaved DDW in a 250 mL conical flask and autoclaved at 15 lbs / inch^2 pressure for 20 min. In another conical flask, 2 g of BSA was dissolved in 22 mL of DDW by gentle stirring on a magnetic stirrer, with care taken to avoid foaming. The albumin fatty-acid supplement was made by adding salt solutions, Tween 80 and vitamins (Table 2.2) to the BSA solution. The supplement was filter sterilized with 0.2 µm syringe filter and added to 75 mL of the EMJH base under aseptic conditions. The medium was stored at 4ºC for future use.

Table 2.2 Preparation of albumin fatty acid supplement stock solution

	Preparation of reagent stock solution (in a final volume of 100 mL)	**Vol. of stock solution added to 100 mL of EMJH base (µL)**
1	1 g of $CaCl_2.2H_2O$ + 1 g of $MgCl_2.6H_2O$	150
2	0.4 g of $ZnSO_4.7H_2O$	100
3	0.3 g of $CuSO_4.5H_2O$	10
4	0.02 g of Vitamin B12*	200
5	1.1 g of Sodium pyruvate	363

6	10 mL of Tween 80**	1250
7	10 mL of Glycerol	100

*All the solutions except vitamin B12 were autoclaved and stored as aliquots at – 20°C.

**Tween 80 was always prepared freshly before use.

2.3.3. Iron solutions

Preparation of stock solution (5 mg Fe / mL): 2.5 g of $FeSO_4.7H_2O$ (Merck) was dissolved in 5 mL of 1 N H_2SO_4 in a 100 mL volumetric flask. 20 mL of 0.01 N H_2SO_4 was added and the solution was warmed till the salt dissolved completely. The solution was made up to 100 mL with glass DDW and aliquoted as 10 mL in McCartney bottles and autoclaved at 15 lbs / $inch^2$ for 20 min.

200 µL of the solution added to 100 mL of liquid EMJH-BSA media to get a final concentration of 10 µg Fe / mL.

2.4. Maintenance and growth of *Leptospira*

Leptospiral serovars were maintained in EMJH semi-solid medium in screw-capped test tubes at 30°C. The cultures were regularly sub-cultured after an interval of 25-30 days. Growth was monitored regularly under dark field microscope. The organisms were grown in liquid EMJH medium for use in MAT.

Growth of *Leptospira* spp. in high- and low-iron conditions was standardized as follows. All glassware was made iron free by soaking with 2% methanolic KOH overnight (o/n) followed by soaking in 8N HNO_3 o/n and subsequent washes with double distilled water. Leptospires were initially grown in regular EMJH liquid medium at 30°C until the culture reaches mid log phase (3×10^8 to 6×10^8 cells / mL), and the bacteria were pelleted (6,000 x *g*, 15 min, RT), washed in 10 mL of EMJH base (Difco, MD, USA), and resuspended in 1 mL EMJH base. These washed cells were inoculated into EMJH BSA high Fe (HI) media containing 10 µg Fe mL^{-1} or EMJH BSA low Fe media where the total iron content in the media was reduced by addition of iron chelator EDDA (200 µM / 400 µM) followed by incubation of media at 37°C, o/n. The leptospiral cells were grown in the respective media for a period of 7-10 days before harvesting for further analysis.

2.5. Analysis of leptospiral proteins

2.5.1. Preparation of whole-cell sonicates

The cultures were harvested at 11,000 x *g* for 20 min, washed thrice with 10 mM Tris-HCl (pH 8.0) and were resuspended in same buffer and sonicated for about 5 min (20 sec pulse at 20 Hz in Vibra cell sonicator, USA). 1% SDS was added to the sonicated samples and incubated at 37°C o/n. Samples were centrifuged at 11,000 x *g* for 10 min to remove the cell debris.

2.5.2. Estimation of protein concentration by Bicinchoninic acid (BCA) method

Bicinchoninic acid (BCA) protein assay kit (Sigma) was used to determine the protein concentration of cell lysates / purified proteins as per manufacturer's instructions. Briefly, serially diluted BSA standards (200-1,000 µg / mL) and 5 µL of protein sample whose total volume was made to 25 µL with DDW was added to the microplate. 200 µL of BCA reagent (196 µL of BCA + 4 µL of 4% (w / v) $CuSO_4 . 5H_2O$) was added to each well and the plate was incubated for 30 min at 37°C. Colour development was measured at 570 nm using an ELISA reader (Model 680XR; Bio-Rad, CA, USA). The concentration of protein present in the sample was interpolated from the BSA standard curve.

2.5.3. Separation of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

Stock solutions

i) Acrylamide and N, N'-bisacrylamide mix (30:0.8): 30 g of acrylamide and 0.8 g of bisacrylamide were dissolved in 60 mL of water and made up to 100 mL.
ii) Resolving gel buffer: Tris-HCl (1.5 M, pH 8.8) with 0.4% SDS.
iii) Stacking gel buffer: Tris-HCl (0.5 M, pH 6.8) with 0.4% SDS.
iv) Ammonium per sulfate (APS): 10% APS solution was prepared fresh in DDW.
v) Sample buffer (2X): 0.125 M Tris-HCl (pH 6.8) containing 4% SDS, 20% glycerol and 0.002% bromophenol blue.
vi) Running buffer: Tris-glycine buffer (25 mM Tris, 250 mM glycine and 0.1% SDS), pH 8.8.

vii) Staining solution: 0.25% Coomassie Brilliant Blue in 50% methanol and 10% glacial acetic acid.

viii) Destaining solution: 10% methanol and 10% glacial acetic acid in DDW.

The resolving gel was prepared either as 5-20% gradient gel or 10% gel (mini-gel) using the recipe as given in Table 2.3 and allowed to polymerize for 40 min. The stacking gel (Table 2.4) was poured over it after appropriately positioning the comb. For gradient gels, 60 µg of total protein mixed in 1:1 ratio with 2x sample buffer was boiled for 10 min and centrifuged for 10 min at 7000 x *g* to remove any insoluble material. The clear supernatant was loaded onto gel and electrophoresis was carried out at 25 mA constant current in the Hoefer electrophoresis unit (Amersham Pharmacia Biotech AB, CA, USA). The gel was allowed to run until the tracking dye had migrated completely off the gel, followed by an additional run for 30 min for optimal resolution. The gel was stained with Coomassie Blue for 1-3 h and then destained overnight.

For mini gels, 30 µg of total protein was loaded and electrophoresis was carried out using Broviga electrophoresis unit (Balaji Scientific Services, Chennai, India).

Table 2.3 Composition of resolving gel

Ingredients	**Volume (mL)**		
	Gradient gel		**10%**
	5%	**20%**	
Acrylamide: Bisacrylamide	2.75	10.60	10.6
Resolving gel buffer	4.0	4.0	8.0
Double distilled water	9.3	1.40	13.4
Ammonium per sulfate	0.08	0.08	0.16
TEMED	0.008	0.008	0.008
Total volume	15	15	30

Table 2.4 Composition of stacking gel

Ingredients	**Volume (mL)**
Acrylamide: Bisacrylamide	1.5

Stacking gel buffer	2.5
Double distilled water	6.0
Ammonium per sulfate	0.03
TEMED	0.01
Total volume	10.04

2.5.4. Western blot analysis

Stock solutions

i) Transblot buffer (10X): Stock solution was prepared by dissolving 250 mM Tris and 1.3 M glycine in 400 mL of DDW. Working solution was prepared by mixing 200 mL of 10X stock solution and 400 mL of methanol in 1.4 L of DDW.
ii) Tris-buffered saline (TBS): 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl.
iii) TBST: TBS containing 0.05% Tween 20.
iv) Ponceau S stain: Ponceau S, trichloroacetic acid and sulfosalicylic acid were mixed in 2:30:30 (w / v) ratio and the final volume was made up to 100 mL with DDW. One part of stock solution was diluted with 9 parts of deionized water to make a working solution.

Electrophoretic protein transfer (Towbin *et al.*, 1979)

Proteins fractionated on polyacrylamide gels were electrophoretically transferred on to nitrocellulose membrane at 30 V constant voltage overnight or 60 V for 2½ h using 1X transblot buffer in Broviga transfer apparatus (Balaji Scientific Services, India).

Development of blot

After the transfer, the membrane was removed and stained with Ponceau S stain to visualize the transferred proteins; the position of the protein molecular weight marker bands were marked with pencil. The membrane was blocked for 2 h using 5% non-fat milk solution (NFM) dissolved in TBST. The membrane was washed thrice with TBS and incubated o/n with appropriate hybridoma culture supernatant

/ antiserum diluted in TBST containing 1% NFM at 4°C. The membrane was washed 4 times with TBST and incubated with appropriate enzyme-conjugated secondary antibody at room temperature for 1½ h. Then, the blot was washed thoroughly with TBS. A ready to use 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium solution (BCIP-NBT; Sigma) was used as a chromogenic substrate for ALP-conjugated blots, while peroxidase-conjugated blots were developed using DAB (diaminobenzidine)-H_2O_2 solution (10 mg DAB in 15 mL TBS with 0.08% H_2O_2).

2.6. Evaluation of diagnostic potential of the hemin-binding protein HbpA

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

The antigens used in MAT were 6-8 day old live cultures of the serovars as mentioned in Table 2.1. All the cultures, grown in liquid EMJH enrichment medium was diluted to obtain density equivalent to McFarland I. Care was taken to ensure that there was no clumping of the organisims.

The serum samples (sources indicated below in the respective sections in ELISA) were serially diluted to obtain upto 1:3200. This was done as follows: 50 µL of phosphate buffer saline (PBS), pH 7.2 was added to all the 96 wells of round-bottom microtiter plate (Tarsons, India). Row A served as a negative control and an additional 48 µL of PBS was added to wells of row B. 2 µL of serum was added to all wells of row B such that the final dilution of the serum is 1:50. The latter was then serially diluted from row B to row H using a multi-channel pipette.

50 µL of the live leptospiral serovars were added to each well. The plate was gently shaken for 15-20 s to mix the contents, covered to prevent any contamination and evaporation, and incubated for three hours at 37°C. A drop from each well was placed onto a glass slide and examined by dark-field microscopy at 40x magnification (Nikon Eclipse E600, Japan) for agglutination or clumping. A serum sample was considered positive when 50% or more organisms were agglutinated and the dilution at which this occurred was taken as the titer.

2.6.2. Preparation of $HbpA_{55}$ antigen

The 1,449 bp *hbpA* fragment encoding amino-acid residues from 228-711 was cloned and expressed (referred to as $HbpA_{55}$) in pET-28a (+) as described by Asuthkar *et al.*, 2007. The purification of the recombinant protein under denaturing conditions using Ni-NTA His-bind columns (Novagen) was done as per protocol in section 2.6.2.1.

2.6.2.1. Affinity purification of $HbpA_{55}$ using Ni-NTA column

Preparation of solutions

i) Charging buffer: 1.05 g of $NiSO_4$ (50 mM) was dissolved in 95 mL of DDW and the volume made up to 100 mL.
ii) Equilibration buffer: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjusted pH to 8.0 with NaOH.
iii) Wash buffer A: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjusted pH to 6.3 with HCl.
iv) Wash buffer B: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjusted pH to 5.9 with HCl.
v) Elution buffer: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjusted pH to 4.5 with HCl.

Purification of the recombinant proteins was done using Ni-NTA His-bind resin under denaturing conditions. A column with 5 mL bed volume of Ni-NTA His-bind resin was packed and charged by passing 6 bed volumes charge buffer. The resin was then equilibrated with 5 bed volumes of equilibration buffer before loading the sample. The soluble supernatant was loaded onto the column which was securely capped and the His-tagged proteins were allowed to bind to the resin for 1 h at 4°C with end-over-end mixing. The column was washed extensively with 5 bed volumes of wash buffer A and wash buffer B. The bound protein was then eluted with elution buffer and collected as 1 mL fractions. The different fractions were analysed by SDS-PAGE (Laemmli, 1970) and fractions containing the pure recombinant protein were pooled and concentrated using Amicon centrifugal filter (3000 MWCO, Millipore, Darmstadt, Germany).

2.6.3. Determination of cross-reactivity of anti-HbpA antibodies with other bacterial antigen

The polyclonal antibodies against HbpA protein were prepared as reported previously (Asuthkar *et al.*, 2007). The polyclonal antibodies were tested for cross-reactivity against whole-cell sonicates of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris*, *Klebsiella pneumonia* (collection of the L. V. Prasad Eye Institute, Hyderabad, India) and *Mycobacterium tuberculosis* (ATCC 27294, lab collection). The plates were coated with 500 ng / well of cell-free sonicates and anti-HbpA antibody was added at 1:100 dilution. Duplicate sets were done and goat anti-rabbit IgG alkaline phosphatase conjugate (1: 5000 dilution; Bangalore Genei) was added to each set and developed. Purified rHbpA (100 ng) and whole-cell sonicates of *Leptospira interrogans* serovar Manilae, grown under high and low-iron conditions (Asuthkar *et al.*, 2007), were included as controls. The experiment was repeated three times with each reaction set up in duplicate.

2.6.4. Development of indirect ELISA for detection of anti-HbpA antibodies in human serum samples suspected for leptospirosis

2.6.4.1. Source of human serum samples

The human subjects included in this study are detailed in Table 2.5 and include patients (and contacts) attending the Uvea Clinic, Aravind Eye Hospital, Madurai (Group I-V) and Nizam Institute of Medical Sciences (NIMS), Hyderabad (Group VI).

Table 2.5 Human subjects included in the study

Group	No. of cases	Clinical criteria for inclusion
I	60	The subjects were clinically confirmed cases of leptospiral uveitis. The inclusion criteria were acute, anterior or pan, non-granulomatous uveitis. The patients were subjected to detailed clinical history, an extensive review of systems, a complete ophthalmic examination by slit lamp & indirect ophthalmoscopy. All routine laboratory and ancillary tests were done. All other causes of uveitis were ruled out. Based on the results of MAT, 30 seropositive and 30 seronegative cases were included.
	Remarks: Previous leptospiral infection was strongly indicated in patients with hypopyon, disc oedema, vasculitis and vitreous membrane or vitreous exudates.	

	(Priya *et al.*, 2007; Rathinam, 2005).	
II	20	The subjects were clinically diagnosed cases of systemic leptospirosis according to WHO criteria (Rathinam *et al.*, 1997) and were seropositive by MAT.
	Remarks: These patients were referred from nearby hospitals for confirmation of leptospirosis by serodiagnosis.	
III	20	Non-leptospiral uveitis controls: ocular inflammation due to other causes*; all were seronegative by MAT with no evidence of fever within the recent past.
IV	10	Cataract controls: they had no ocular inflammation and all were seronegative by MAT.
V	20	Endemic controls: healthy normal individuals with no evidence of disease and MAT negative.
VI	35	The subjects were clinically suspected cases of systemic leptospirosis.

***** This group includes patients with Vogt Koyanagi Harada Syndrome (4), Posterior Choroiditis (1), Behcet's uveitis (3), Sympathetic Ophthalmia (2), Unilateral granulomatous pan uveitis with Tractional Retinal Detachment (1), Sarcoid uveitis (1), Fuch's heterochromic uveitis (6), Bilateral granulomatous uveitis (1) and HLA B27 related uveitis (1)

2.6.4.2. PanBio IgM ELISA

All the human serum samples were tested for anti-leptospiral antibodies with the commercial PanBio *Leptospira* IgM ELISA (Inverness Medical Innovation Pty. Ltd., Australia) which utilises micro well test strips pre-coated with *L. biflexa* serovar Patoc antigen. 100 µl of serum (diluted 1:100) was added to the microwell strips and incubated for 30 minutes at 37°C. Residual serum was removed by washing and 100 µL peroxidase conjugated anti-human IgM was added, followed by incubation for 30 min at 37°C. After sufficient washes to remove the unbound conjugate, 100 µL tetramethyl benzidine / hydrogen peroxide was added and the plates were incubated for 10 min. The reaction was stopped by the addition of 100 µL of 1 M phosphoric acid and the absorbance was read at 450 / 600 nm using an ELISA reader (Model 680XR; Bio-Rad, CA, USA).

Each set of tests was run with a positive control, negative control and cut-off calibrator in duplicate provided with the kit. The test was valid when the absorbance readings of the above met the specifications of the PanBio instructions. The cut-off value was calculated by multiplying the mean absorbance of the calibrator sample replicates with the calibration factor. The PanBio unit for each sample was calculated by dividing the absorbance of the sample by the cut-off value and then multiplying by 10. A score < 9 units indicated a negative result interpreted as no evidence of recent infection. A score of 9-11 units indicated an equivocal result where the samples should be re-tested. If the samples remained equivocal, testing should be repeated by an alternate method or another sample should be collected. A score of > 11 units was interpreted as a positive result, indicating the presence of leptospira-specific IgM antibodies.

2.6.4.3. Human $HbpA_{55}$ ELISA

Preparation of solutions

i) Bicarbonate buffer (pH 9.2): 159 mg of sodium carbonate and 293 mg of sodium bicarbonate were dissolved in 90 mL H_2O. The pH was adjusted to 9.2 with 0.1 N HCl and the volume was made to 100 mL with H_2O. The solution was autoclaved and stored at 4°C.
ii) 0.1 M PBS (pH 7.2): Dissolved 8 g of NaCl, 2.4 g of KH_2PO_4, 14.4 g of Na_2HPO_4 and 2 g of KCl in 900 mL of DDW, pH adjusted to 7.2 with 0.1 N HCl and the volume made upto 1000 mL with H_2O and autoclaved.
iii) PBST: 0.1 M PBS containing 0.05% Tween 20.
iv) Blocking solution: 0.1 M PBS containing 5% BSA.
v) Secondary antibody: Rabbit anti-human IgG alkaline phosphatase conjugate (1 : 5000 dilution; Bangalore Genei), goat anti-human IgM and IgG peroxidase conjugates (1:5000; Sigma Aldrich)
vi) Substrate for ALP-conjugate:
    a. Diethanolamine buffer (DEA): 24.2 mL of diethanolamine (Sigma) was dissolved in 200 mL H_2O and the pH was adjusted to 9.8 with 0.1 N HCl, added 0.5 mM $MgCl_2$ and then made to 250 mL final volume with H_2O.

b. p-nitrophenyl phosphate (pNPP) substrate solution: 1 tablets of p - nitrophenylphosphate (5 mg) was dissolved in 5 mL of diethanolamine buffer to get 1 mg / mL solution.

vii) Substrate for Peroxidase conjugate:

a. 0.05 M phosphate-citrate buffer (pH 5): 5.11 g of citric acid and 7.3 g of disodium hydrogen phosphate was dissolved in 900 mL H_2O. The pH was adjusted to 5 and the volume was made upto 1 L.
b. 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution: 1 mg TMB tablet was dissolved in 10 mL of 0.05 M phosphate citrate buffer and 3 µL of fresh 30% hydrogen peroxide was added prior to use.
c. Stop solution: 1.25 M H_2SO_4.

To optimise indirect ELISA, a checkerboard titration was done to determine the optimal concentration of HbpA antigen and the appropriate dilution of the serum. The microtiter plate was coated with serial 2-fold dilutions of antigen from 2000 ng / well to 31.25 ng / well from wells A to G. A known positive serum sample was serially diluted from rows 2 to 12. The optimal concentration for antigen coating was chosen as the concentration that gave a signal greater than 1.0 and allowed the highest signal-to-noise ratio.

For $HbpA_{55}$ ELISA, flat-bottomed polystyrene microtitre plates (Corning Inc., Corning, New York, USA) were coated with 250 ng rHbpA (100 ml in 0.1 M bicarbonate buffer, pH 9.2) for 3 h at 37°C in a humid box, followed by overnight incubation at 4°C. The plates were washed three times with PBS-T and incubated with a blocking solution for 2 h at 37°C. After washing, 100 µL of human serum (1:200 dilution) was added to the wells. After an incubation for 2 h at 37°C, the plates were again washed three times with PBS-T and 100 µL of enzyme-conjugated secondary antibody were added to the wells and the plates were incubated for 2 h at 37°C. Color development for the ALP conjugates was done by adding 100 µL of pNPP substrate solution, incubated at room temperature for 15 min and the absorbance at $OD_{405\ nm}$ was read in an ELISA reader (Model 680XR; Bio-Rad, CA, USA). For peroxidase-labeled conjugates, colour was developed in the dark with freshly prepared TMB substrate solution and the reaction was stopped using 1.25 M sulphuric acid after 10 min. The absorbance at $OD_{450\ nm}$ was read with an ELISA reader.

Antigen and antibody blanks were included in the test to check for background absorbance. A known positive serum sample and a serum sample from a healthy individual were used as positive and negative controls, respectively.

2.6.5. Screening for anti-HbpA$_{55}$ antibodies in bovine serum samples suspected for leptospirosis

2.6.5.1. Source of bovine serum samples

176 bovine serum samples collected from various geographical regions of India were obtained from Indian Veterinary Research Institute, Izatnagar, India (a kind gift from Dr. S. K. Srivastava).

2.6.5.2. Preparation of rLipL41 and rLipL32 antigen

Bovine HbpA-ELISA was also compared with ELISA using the leptospiral antigens LipL41 and LipL32, whose diagnostic potential was established by other studies (Bomfim *et al.*, 2005; Mariya *et al.*, 2006; Senthilkumar *et al.*, 2010). The genes encoding *lipL41* and *lipL32* were cloned into the bacterial expression vector pRSET A (Invitrogen, USA), transformed into BL21(DE3) pLysS (Invitrogen, USA), induced using 1 mM isopropyl β-D thiogalactoside (IPTG) and expressed as C-terminal 6xHis extensions with molecular weights of 41 kDa and 32 kDa respectively. The recombinant proteins were purified under denaturing conditions using commercial Ni-NTA His-bind columns (Novagen) as per section 2.6.2.1.

2.6.5.3. Bovine HbpA$_{55}$ (IgG) ELISA

ELISA was performed as mentioned in section 2.6.4.3. The plates were coated with 250 ng / well of rHbpA and 50 ng / well of rLipL41 and rLipL32 and bovine serum samples were added at 1:200 dilution. Triplicate sets were done and rabbit anti-bovine IgG alkaline phosphatase conjugate (1:500 dilution; Bangalore Genei, India) was added to each set and developed as mentioned. A known positive serum sample and normal bovine serum were used as positive and negative controls respectively.

2.7. Statistical analysis

Statistical analysis of ELISA was carried out using GraphPad Prism software (Version 5.01, GraphPad Software, Inc., San Diego, CA) and SPSS version 13 statistical package (SPSS Inc., IL, USA). The results were analysed by One-way ANOVA tests and considered significant when $P < 0.05$.

The cut-off value for $rHbpA_{55}$ ELISA with human serum samples was calculated by adding two times standard deviation (SD) to the mean value of normal endemic controls (2SD + Mean). The relative sensitivity, specificity and accuracy of the test were determined using MAT as the gold standard.

$$\text{Sensitivity} = [a / (a + c)] \times 100;$$

where a = number of serum samples positive by the ELISA and MAT; c = number of serum samples positive by MAT but negative by ELISA.

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

where d = number of serum samples negative by ELISA and MAT; b = number of serum samples negative by MAT but positive by ELISA.

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

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

$$\text{Positive predictive value} = a / (a + b) \times 100$$
$$\text{Negative predictive value} = d / (c + d) \times 100.$$

A receiver operating characteristic curve (ROC) (MedCalc Software, Mariakerke, Belgium) (Schoonjans *et al.*, 1995) was used to calculate the cut-off value, sensitivity, specificity, area under curve (AUC), 95% CI (confidence intervals) and others to determine the diagnostic value of an antigen for diagnosis of bovine leptospirosis.

Kappa statistics (Altman, 1990) was used to assess the degree of agreement of HbpA-ELISA with LipL41-ELISA and LipL32-ELISA and the κ values obtained were used to grade the performance as follows: poor (0.00-0.20), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80) and very good (0.81-1.00).

2.8. Generation of monoclonal antibodies against HbpA of *L. interrogans* serovar Lai and evaluation of their potential in antigen detection in serum samples

2.8.1. Preparation of $HbpA_{55}$ antigen

Preparation of solutions

i) Elution buffer: 25 mM Tris-HCl (pH 8.8) buffer containing 5% glycerol, 1% SDS and 0.24 mM glycine.
ii) Dissolving buffer: 25 mM Tris-HCl (pH 8.8), 0.6% SDS.

The insoluble inclusion bodies containing the expressed $rHbpA_{55}$ protein (section 2.6.2) was separated on 10% preparative SDS-PAGE. $HbpA_{55}$ protein bands were excised and crushed with the help of a gel crusher, and resuspended in elution buffer and incubated at 37°C. The eluted protein was subjected to acetone precipitation at -20°C and the dried pellet was dissolved in minimum amount of dissolving buffer.

2.8.2. Development of monoclonal antibody against HbpA

2.8.2.1. Immunization of mice with HbpA

BALB/c female mice between 5-6 weeks of age were purchased from National Institute of Nutrition (Hyderabad, India). All animal studies was reviewed and approved by Institutional Ethics Committee (IEC), Indian Immunological Ltd., Hyderabad.

The mice were immunized with HbpA for the generation of immunogen-primed splenocytes to be used in cell fusion experiment as per schedule employed listed in Table 2.6.

HbpA was emulsified with the adjuvant thoroughly using a syringe and needle and incubated at 4°C for 15 min. If the layers separated out, the emulsification process was repeated. The emulsion was tested by placing a drop of emulsified antigen on the surface of cold water in a beaker and was found satisfactory if the drop remained intact without dispersing. The emulsion was then injected into the peritoneal cavity of BALB/c mice. Three days prior to fusion, a

booster without adjuvant was given intravenously via the tail vein. The mice were test bled via the retro-orbital venous blood route on days 14, 21, 56 to check for antibody titers by indirect ELISA and immunoblotting.

Table 2.6 Immunization schedule of BALB/c mice

Day	Immunisation	Adjuvant	rHbpA (µg)	Volume (mL)	Route of immunisation
0	Primary	Freund's complete	100	0.2	Intra-peritoneal
14	I booster	Freund's incomplete	50		
21	II booster				
	Intra-venous pre-fusion boosters				
54	I booster	Nil	10	0.1	Intra-venous
55	II booster				
56	III booster				

2.8.2.2. Collection of blood and serum

Chloroform was used to mildly sedate the mice to be bled. Blood was obtained from retro-orbital venous plexus by inserting a microhematocrit tube into the medial canthus of the eye. Using gentle pressure, the tube was twisted until it entered the orbital venous plexus. The tube was tilted downward to allow the blood to flow smoothly into a micro-centrifuge tube. Blood was allowed to coagulate at room temperature for 1 h and overnight at 4°C, then centrifuged for 10 min at 7000 x *g* and the serum fraction (supernatant) transferred into a new tube and stored at -20°C.

2.8.2.3. Preparation of cell-culture growth media

Media for use in myeloma and hybridoma cell culture was prepared under aseptic conditions as given in Table 2.7.

Table 2.7 Cell culture media composition

Component	TCGM*	TCGM + 8-Azaguanine	TCGM HAT	TCGM HT	TCGM (Antibiotic free)
	(mL)				
DMEM	77	75	75	75	78

FBS	20	20	20	20	20
8-Azaguanine	-	2	-	-	-
HAT (50X)	-	-	2	-	-
HT (50X)	-	-	-	2	-
L-Glutamine (200 mM)	2	2	2	2	2
Antibiotic and Antimycotic solutions	1	1	1	1	-

* - Tissue culture growth media

After mixing the components, the different media were sterilised by filtration through 0.45 μm filter and stored at 4°C till further use.

2.8.2.4. Preparation of Sp2/0 myeloma cells

Sp2/0 cells were thawed at least 15 days in advance from liquid nitrogen, and cultured in TCGM-antibiotic free media. The cells were then passaged four times in TCGM with 8-azaguanine for selecting $HGPRT^-$ mutants. The Sp2/0 cells were then passaged in fresh TCGM medium every 24 hours for 3 days before fusion. On the day of fusion, the Sp2/0 cells were harvested, and washed twice in TCGM without serum. The cell viability was determined by trypan blue exclusion using a haemocytometer. Cell suspensions with > 90% viability were selected and resuspended in 10 mL of TCGM.

2.8.2.5. Isolation of mouse splenocytes

Each immunized mice was killed by cervical dislocation 3 days after the final booster and immersed in a beaker containing 70% ethanol and laid out on a dissecting board. The spleen was removed aseptically from the mouse, and placed in a universal bottle containing 10 mL of serum-free TCGM. After carefully dissecting away surface fat and other adhering tissue, the spleen was washed four times in TCGM and transferred to sterilized cell strainer. A sterile 10 mL syringe plunger was used to tease the spleen in a cell strainer to obtain single-cell suspension of splenocytes. The cell suspension was collected in a 15 mL tube,

and left for 2 min at room temperature to allow the larger tissue pieces to settle to the bottom. The top 95% of the cell suspension was transferred to a new 15 mL centrifuge tube, and centrifuged at 170 x *g* for 7 min. The cells were washed once with TCGM. The contaminating RBC was removed by resuspending the pellet in 5 mL of sterile RBC lysing solution (Sigma, USA). The cells were incubated in room temperature for 5 min to which 7 mL of FBS was added and centrifuged at 170 x *g* for 7 min. The cells were then washed twice with TCGM and resuspended in 10 mL of TCGM. A viable cell count was performed as described above.

2.8.2.6. Hybridoma fusion

Two hybridoma fusions were concurrently performed from B cells obtained from two immunized mice. A viable cell count of splenocyte and myeloma was done and the cells were mixed in a ratio of 1:10 into a 50 mL conical centrifuge tube. The cells were centrifuged for 7 min at 400 x *g,* the supernatant removed completely and the cells in the pellet was disturbed by gently tapping the bottom of the tube. Fusions were performed at 37°C temperature in water-bath. One mL of 50% PEG 4000, pre-warmed to 37°C, was added to the cells over a period of 30 sec with continual stirring. Stirring of the cells was continued for 1 additional min. 32 mL of DMEM medium were then added slowly over 11 min with continual stirring. The cells were incubated for 5 min and subsequently centrifuged for 5 min at 400 x *g*. The supernatant was discarded and the cells were gently resuspended into TCGM-HAT media. One-third of the cells were seeded into seven 96 well tissue culture plates with feeder culture (mouse thymocytes). The remaining two - third of the fused hybrids were cryopreserved in liquid nitrogen (section 2.8.3). The hybridoma cells were monitored every 5 days, 100 µL of spent media was removed and topped up with fresh TCGM with 1X HAT for 4 times and TCGM with 1X HT 2 times after which media change and passage was done with TCGM only. The cells were tested for MAb specificity to HbpA protein using indirect ELISA (refer section 2.8.2.7.) when 50% confluency was reached. The positive hybridomas were passaged for further processing.

2.8.2.7. Screening for positive hybridomas

Screening for antibody producing hybridoma clones was started after 7 days of incubation.

Screening by ELISA

Initial screening for antibody producing hybridoma cells was done by indirect-ELISA (protocol described in section 2.6.4.3.). The plates were coated with 200 ng / well of rHbpA and were blocked with 1% bovine gelatin in PBS. Cell culture supernatant was aseptically added to antigen coated ELISA plates using an 8 channel pipette such that the ELISA plate was an exact replica of culture plate. Care was taken not to spill contents while pipetting and the pipette tips were changed for every well to avoid cross-contamination from other monoclonal antibodies. Anti-Mouse IgG (Fc specific)-peroxidase antibody (dilution 1: 25,000, Sigma, USA) was added to each well and developed as mentioned before. *E. coli* BL21 (DE3) cell lysate and purified recombinant His-tagged enterotoxin from *Clostridium* spp. and LipL32 proteins were used as negative antigen controls.

2.8.2.8. Single cell cloning by limiting dilution technique

The hybridomas which showed positive reactivity against HbpA antigen in the initial screening were subjected to single cell cloning by limiting dilution technique. This was done by serially diluting the positive hybridoma to concentration of 10^2 cells / mL. The final suspension was made upto 7 mL with TCGM in a petridish. The cells were seeded as 100 µL / well in a T_{96} well plate in the internal 60 wells with feeder layer (2 x 10^4 thymocytes) and incubated in a CO_2 incubator at 37°C. On day 4, each well was screened for the number of growing colony / colonies present after which the media was changed by removing 100 µL of spent media and topped with 100 µL of fresh TCGM. When the size of the cell colony increased, it was disturbed gently during media change by multichannel pipette to avoid piling up which may lead to cellular suffocation and death. When the clone attained 50% confluence, 100 µL of spent medium was harvested and checked for antibody secretion as described above. The process was repeated with the highly reactive monoclones (second limiting dilution) and checked for antibody secretion. The highly reactive clones were subjected to further characterization and the low reactive clones were labeled and cryopreserved.

2.8.3. Cryopreservation of cells

Cell lines and positive hybridomas in log phase were harvested at 170 x *g* for 7 min. The pellet was resuspended in pre-chilled freezing solution (90% FBS, 10% DMSO) with a count of 5-10 x 10^6 cells / mL per vial. The vials were stored in isopropanol freezing box at -70°C for atleast 20 hours. The next day the tubes were transferred into liquid nitrogen containers.

2.8.4. Revival of cells

The cryotubes were surface sterilized with 70% alcohol and the frozen cells were thawed quickly in a water bath (37°C). The cell suspension was transferred to a 15 mL centrifuge tube and diluted with 10 mL of TCGM. The cells were harvested by centrifuging at 170 x *g* for 10 min. The supernatant was decanted and the cell pellet was resuspended in 10 mL of TCGM antibiotic-free media and seeded in 25 cm^2 tissue culture flask. The flasks were incubated at 37°C in humidified CO_2 (5%) incubator.

2.8.5. Characterization of Monoclonal antibodies

2.8.5.1. Immunoglobulin subclass and isotype determination

Immunoglobulin isotypes of MAbs was determined by capture ELISA using Pierce Rapid ELISA Mouse mAb isotyping kit (Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's instructions. The assay uses ELISA strip-well plates with individual wells pre-coated with anti-mouse heavy-chain capture antibody (anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgM) or anti-mouse light-chain capture antibody (kappa or lambda). Briefly, the hybridoma supernatants were diluted 1:50 in TBS and added to each well of pre-coated 8-well strip. Goat anti-mouse HRP conjugate was added and the strips were incubated for 1 hour at room temperature. After washing TMB substrate was added to each well and development of blue colour was recorded as positive.

2.8.5.2. Cross-reactivity of MAbs with other bacterial antigens

Cross-reactivity of MAbs with other bacterial antigens was done as mentioned in section 2.6.3. DMEM media and PBS were used as antibody controls in the assay.

2.8.5.3. Antigenicity of HbpA: Determination of immunoreactivity of MAbs with three antigenic fragments of HbpA

The three antigenic fragments of HbpA: $HbpA_{20}$ (amino-acid residues 110-271), $HbpA_{25}$ (266-454), $HbpA_{34}$ (421-711) were PCR amplified from genomic DNA of *L. interrogans* serovar Lai. The three PCR amplicons $hbpA_{484}$, $hbpA_{566}$ and $hbpA_{871}$ were cloned into pET28a (+) to generate recombinant plasmids. The plasmids were transformed into *E. coli* BL21 (DE3) for expression of the recombinant protein. The proteins were purified and used to determine its immunoreactivity with MAbs by ELISA and immunoblotting.

Standard molecular biology techniques were used unless otherwise stated (Sambrook *et al.*, 1989).

2.8.5.3.1. Preparation of genomic DNA from *L. interrogans* serovar Lai

Preparation of solutions

i) Tris-EDTA buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
ii) Lysozyme (10 mg / mL stock): 10 mg of lysozyme was dissolved in 1 mL DDW and stored at - 20°C
iii) Proteinase K (10 mg / mL stock): 10 mg of proteinase K was dissolved in 1 mL DDW and stored at - 20°C.
iv) 10% SDS: 10 g of SDS was dissolved in 100 mL DDW.
v) 5 M NaCl: 29.2 g of NaCl was dissolved in 60 mL of water and made up to 100 mL.
vi) Chloroform: isoamylalcohol (24:1): Mix 24 volumes of chloroform with 1 volume of isoamyl alcohol. Stored at 4°C.

Leptospiral genomic DNA was isolated using published protocol (Narayanavari *et al.*, 2012b). 10 mL culture was harvested at 7000 x *g* for 15 min and washed thrice with TE buffer. The pellet was resuspended in 400 µL of TE buffer and incubated with 50 µL of lysozyme for 1 h at 37°C. The clear solution was treated with 6 µL of proteinase K, 70 µL of 10% SDS and incubated in a water bath at 50°C for 30 min. 100 µL of 5 M NaCl was added, mixed gently and the contents were transferred to Phase lock gel tube (Eppendorf, Germany). 750 µL of chloroform: isoamyl alcohol mixture (24:1 v / v) was added, centrifuged at 12000 x

g for 5 min and the upper aqueous layer was transferred into a fresh tube. The genomic DNA was precipitated by the addition of an equal volume of isopropanol, allowed to stand for 30 min at room temperature, followed by centrifugation at 12,000 x *g* for 15 min. The DNA pellet was washed with 1 mL of 70% ice-cold ethanol. The pellet was air dried at room temperature and dissolved in TE buffer. DNA concentration was estimated spectrophotometrically using the Nano Drop spectrophotometer (Thermo Scientific, USA) by measuring the absorbance at 260 nm and 280 nm.

2.8.5.3.2. Agarose gel electrophoresis

Preparation of solutions

i) Tris-acetate-EDTA (TAE) buffer: Dissolved 4.84 g Tris-HCl, 1.14 mL glacial acetic acid and 20 mL 0.5 M EDTA in 1 L of DDW and pH was adjusted to 8.0.
ii) Ethidium bromide: 1 mg / mL stock solution was prepared and added to the gels to give a final concentration of 0.1 µg / mL.

One volume of DNA to be separated were mixed with 5 volumes of 6x sample buffer and subjected to separation in a 0.8-1% agarose gel in a horizontal agarose gel unit (Balaji Scientific Services, India) at 80 to 100 V for approximately 1-1.5 h. After electrophoresis the DNA fragments were visualised in a UV transilluminator (UVP GelDoc, Cambridge, UK).

2.8.5.3.3. *E. coli* strains and plasmid vector

The *E. coli* strains and plasmids used for the study are as listed in Table 2.8.

Table 2.8 *E. coli* strains and plasmids

Strain or Plasmid	Description	Reference or source
E.coli strains		
DH5α	Cloning host	Lab collection
BL21 (DE3)	Expression host	Novagen
Plasmid		
pET28a (+)	*kan*^{R}, Cloning vector	Novagen, Merck, Darmstadt, Germany

2.8.5.3.4. PCR amplification

PCR amplification of *hbpA* fragments (*hbpA*$_{484}$, *hbpA*$_{566}$ and *hbpA*$_{871}$) was done in a 50 µL volume containing 5 µL of 10X Dream Taq buffer, 10 mM dNTP mix (Fermentas, USA), 10 pmol each of forward and reverse primers (Table 2.9), 1 unit of Dream Taq DNA Polymerase and 200 ng of leptospiral genomic DNA. Amplifications were carried out in the thermal cycler (Eppendorf Master Cycler gradient, USA), using an initial denaturation for 3 min (95°C) followed by 30 cycles of amplification (3 min at 68°C & 15 sec at 95°C) and a final extension for 3 min at 72°C. A negative control without template DNA was included. The PCR products were separated in 1% agarose gel, visualised in a UV transilluminator and purified using Qiagen PCR purification kit as per manufacturer's instructions.

Table 2.9: List of primers

Gene	Sequence (5' to 3')*
hbpA$_{484}$	For – GGGAATTCCATATGAGTGGCGCTAGAAATGCTGC
	Rev – CCCAAGCTTGATAGGCGTTCCCAGTAGTC
hbpA$_{566}$	For – GGGAATTCCATATGGGGAACGCCTATCAAGAC
	Rev – CCCAAGCTTACGGGCTGCAAGTTTAGGAG
hbpA$_{871}$	For – GGGAATTCCATATGTGGACCGTTTCTCGTTCTC
	Rev – CCCAAGCTTTTAAAAGTGGGCCGAGAATC

* For (forward primer) and Rev (reverse primer) containing *NdeI* and *HindIII* restriction sites are underlined respectively.

2.8.5.3.5. Restriction digestion

1 µg of pET28a (+) plasmid DNA and 1 µg of *hbpA*$_{484}$, *hbpA*$_{566}$ and *hbpA*$_{871}$ amplicons were individually subjected to double digestion with 10 units each of *NdeI* and *HindIII* in a reaction mixture of 50 µL and incubated at 37°C for o/n. After heat inactivation at 65°C for 10 min, they were purified using Qiagen reaction clean-up kit as per manufacturer's instructions.

2.8.5.3.6. Ligation

Ligation was done using vector and insert DNA in molar ratio of 1:3 using the following formula.

$$\text{Concentration of the insert (ng)} = \left[\frac{\text{ng of vector x Size of the insert (bp)}}{\text{Size of the vector (bp)}} \right] \text{x } 3$$

The ligation reaction mixture in a volume of 20 µL contained 120 ng of vector DNA, insert DNA (32 ng of *hbpA*$_{484}$, 48 ng of *hbpA*$_{566}$, 73 ng of *hbpA*$_{871}$), 2 µL of 10X ligase buffer, 1 µL of T4 DNA ligase (5 U / µL) and made up to 20 µL with sterile DDW. This was incubated at 16°C o/n , heat inactivated at 65°C for 10 min and stored at -80°C till used. 10 µL of ligation mixture was taken to transform *E. coli*.

2.8.5.3.7. Transformation

Single colony of *E. coli* was inoculated in 5 mL of LB broth under aseptic conditions and incubated o/n at 37°C in an orbital shaker. 1 mL of the culture was used to inoculate 100 mL of LB broth and incubated till $A_{600\ nm}$ reached 0.4. The cells were then harvested by centrifugation at 6000 x *g* for 15 min at 4°C. The cell pellet was re-suspended in 20 mL of ice-cold 100 mM $CaCl_2$ and incubated in ice for 30 min. The cell suspension was centrifuged as before and the competent cells were finally resuspended in 2 mL of ice-cold 100 mM $CaCl_2$ and placed on ice.

10 µL of ligation mixture was added to 200 µL of competent cells, mixed gently and incubated on ice for about 30 min. The cells were then subjected to heat shock at 42°C in a water bath for 90 sec and immediately chilled on ice. 800 µL of warm (37°C) LB broth was added to each tube, incubated with shaking at 37°C for 1 hour, then plated on LB agar plates supplemented with kanamycin (30 µg / mL). The plates were incubated at 37°C o/n.

2.8.5.3.8. Screening of transformants

2.8.5.3.8.1. Plasmid DNA preparation (Birnboim & Doly, 1979)

Preparation of solutions

i) Solution I: 50 mM glucose in 25 mM Tris-HCl (pH 8.0) containing 10 mM EDTA. The pH was adjusted to 8.0 with 1 N HCl and stored at 4°C.
ii) Solution II: 0.2 N NaOH solution containing 1.5% SDS.

iii) Solution III: 5 M potassium acetate (pH 5.2), glacial acetic acid and DDW in ratio of 60.0:11.5:28.5 (v / v).

A single colony was inoculated into 5 mL of LB medium with kanamycin (30 µg / mL) and incubated overnight with shaking at 37°C. 1.5 mL of the culture was centrifuged for 10 min at 7000 x *g* at 4°C, supernatant discarded and the cell pellet was resuspended in 100 µL of Solution I by vortexing and incubated on ice for 5 min. 200 µL of freshly prepared Solution II was added, mixed well and incubated on ice for 5 min. The solution was neutralized by adding 150 µL of ice-cold Solution III, mixed by gentle inversion five times and after incubation in ice for 5 min, centrifuged at 7000 x *g* for 10 min at 4°C. The supernatant was carefully transferred to a fresh tube and DNase free RNase was added to a final concentration of 10 µg / mL and incubated at 37°C for 30 min. The plasmid DNA was extracted into the aqueous phase by the addition of equal volume of phenol:chloroform (1:1) followed by centrifugation at 9600 x *g* for 10 min. The plasmid DNA was precipitated with 2 volume of isopropanol at room temperature for 10 min and centrifuged at 9600 x *g* for 15 min. The pellet was washed with 70% ethanol, air-dried, and dissolved in 20 µL of TE buffer and stored at –20°C.

2.8.5.3.8.2. Verification of recombinant clones

Six to ten transformed colonies were picked and inoculated into 5 mL of LB medium containing kanamycin (30 µg / mL) and incubated o/n at 37°C with shaking. Plasmids were isolated as described above and subjected to restriction digestion (as detailed in results). The digested fragments were analysed by agarose gel electrophoresis.

The recombinant plasmids from the positive colonies were futher confirmed by DNA sequencing (Eurofins Genomics India Pvt. Ltd., Bengaluru, India). Sequence data were analyzed with ChromasPro (Version 2.4, Technelysium Pty. Ltd., Australia).

2.8.5.3.9. Expression of HbpA fragments in *E. coli*

Recombinant plasmid from a positive clone was isolated and transformed into *E. coli* BL21 (DE3) cells for expression studies. A single colony was inoculated in 5 mL of LB medium supplemented with kanamycin (30 µg / mL) and incubated at

37°C for o/n in an orbital shaker. Hundred mL of LB broth in 500 mL conical flask containing kanamycin (30 µg / mL) was inoculated with 1 mL of overnight culture and incubated at 37°C until the absorbance at 600 nm was 0.5 (approximately 3 h). An aliquot of culture representing uninduced cells was transferred to a new tube and IPTG was then added to the cells at a final concentration of 1 mM and the cells incubated with shaking for 3 h at 37°C. The uninduced and induced cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C, washed thrice and subjected to sonication for 5 min with pulses of 20 sec via Vibra-cell sonicator (Sonics & Materials Inc., CT, USA). The pellet and supernatant were separated by centrifugation at 7000 x *g* for 20 min and analyzed by SDS-PAGE (Laemmli, 1970) on a 10% polyacrylamide gel.

2.8.5.3.10. Purification of HbpA fragments

All the HbpA fragments were expressed as insoluble inclusion bodies as deduced in a 10% polyacrylamide gel by SDS-PAGE (Laemmli, 1970). The purification of the protein was done as mentioned in section 2.6.2.1.

2.8.5.3.11. Immunoreactivity of three HbpA fragments with MAb against HbpA

This was done essentially by ELISA and western blotting using procedures described earlier.

2.8.5.4. Sandwich ELISA: Determination of diagnostic potential of MAb for antigen detection in clinical samples

2.8.5.4.1. Determination of lowest concentration of antigen in HbpA-spiked serum sample

The sensitivity of the sandwich ELISA was determined by measuring the lowest amount of the antigen that can be detected by the MAbs. Polyclonal rabbit anti-HbpA antibody was used as antigen-capturing while MAbs 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C as antigen detecting antibody. For each step, 100 µL / well was added unless mentioned otherwise. Polystyrene microtiter plates (Corning, USA) were sensitized overnight at 4°C with antigen-capturing antibody (dilution 1:5000 in 0.1 M carbonate buffer, pH 9.6). The plates were thoroughly washed and

unbound sites were blocked with 300 µL / well of 5% fetal calf serum (Sigma) diluted in PBST, pH 7.4. After 2 h incubation at 37°C, the plates were incubated with a series of two-fold diluted concentrations of purified rHbpA (1000 ng / well to 0.48 ng / well) and whole-cell sonicate of *L. interrogans* serovar Manilae grown under low iron conditions (4000 ng / well to 1.95 ng / well) spiked in FCS and incubated at 37°C for 2 h. Each of the four antigen-capturing MAbs were added individually to the plates. The amounts of bound MAbs were detected as mentioned in section 2.8.2.7.

2.8.5.4.2. Antigen detection in serum of patients with leptospirosis

Sixteen MAT positive serum samples from patients with leptospirosis and 5 MAT negative sera from healthy donors were screened using sandwich ELISA as mentioned above. Serum (1:20 dilution) either alone or spiked with 1 ng of $rHbpA_{55}$ was added to microtiter plate coated with polyclonal rabbit HbpA antiserum. $rHbpA_{55}$ (1 ng / well) served as positive control. MAb 10E3.1C was used as antigen detecting antibody for the assay.

2.9. Identification of a second hemin-binding protein HbpB (LA3149) in *L. interrogans* serovar Manilae

2.9.1. Generation of *hbpA* mutant strain M601 by transposon mutagenesis

hbpA mutant strain M601 (a kind gift from Prof. Ben Adler, Monash University, Australia) generated by random transposon mutagenesis in *L. interrogans* serovar Manilae strain L495 (Murray *et al.*, 2009a) was used for the study. M601 strain was maintained in EMJH enrichment medium with kanamycin at a final concentration of 50 µg / mL.

2.9.2. Confirmation of *hbpA* gene inactivation in mutant

The insertional inactivation of *hbpA* gene was confirmed by PCR using gene-specific primers and the inability of the mutant to express the functional protein was confirmed by immunoblot analysis and whole-cell ELISA using anti-HbpA MAbs.

2.9.2.1. PCR: Confirmation of transposon mediated inactivation of *hbpA*

Genomic DNA isolated from *hbpA* mutant strain M601 and wild type (WT) strain *L. interrogans* serovar Manilae were amplified using the PCR program mentioned in section 2.8.5.3.4. using full length $hbpA_{2149}$ primers (For; 5' – GGGAATTCCATATGTCATCCAACCATTCGATG – 3' and Rev; 5' - CCCAAGCTTTTAAAACTGGGCCGAGAATC) (Asuthkar *et al.*, 2007).

2.9.2.2. Immunoblot analysis: Absence of HbpA in M601 strain

Whole cell sonicates of *hbpA* mutant strain M601 and WT Manilae grown in EMJH BSA media high and low iron conditions (Section 2.4) were analysed for the expression of HbpA protein by 10% SDS-PAGE. The proteins were stained with Coomassie brilliant blue R-250 (Sigma). Western blotting analysis of these proteins was done using 10E3.1C MAb as per protocol mentioned in section 2.5.4.

2.9.2.3. Whole cell ELISA

This was done with 100 µL per well of 10^9 cells / mL of intact WT and *hbpA* mutant strain M601 grown under high and low iron conditions. Prior to coating, the cells were immobilized with 75 mM sodium azide, centrifuged at 1000 x *g*, and resuspended in 0.05 M sodium carbonate buffer (pH 9.6). Plates were blocked with EMJH enrichment media for 3 h at room temperature. MAbs 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C was added to each set and developed as mentioned in section 2.8.2.7. LipL32 antibody was used as a positive control while LipL31 (a cytoplasmic membrane protein) antibody was used as negative control.

2.9.3. Growth of *hbpA* mutant strain M601 in the presence of varying sources of iron

WT Manilae and *hbpA* mutant strain M601 grown till mid-log phase (4 x 10^8 to 6 x 10^8 cells / mL) in EMJH enrichment media were pelleted (4,000 x *g*, 15 min, RT) and washed thrice in EMJH basal media (Difco, USA). The organisms were resuspended at a concentration of 1 x 10^7 cells / mL in EMJH-BSA media in five separate flasks that were supplemented with 10 µg Fe mL^{-1}, 400 µM EDDA, 1 µM hemin chloride (2 flasks preincubated with 400 µM EDDA and 200 µM EDDA) and 1 µM human haemoglobin. Growth was monitored for 10 days by direct counts of bacteria using dark-field microscopy. Three independent experiments were performed and the data was expressed as means ± SD.

2.9.4. Transcriptional regulation of genes in *hbpA* mutant strain M601 and WT Manilae by iron levels

2.9.4.1. Identification of putative Fur-dependent genes: *in silico* prediction of putative Fur boxes in iron-regulated genes

Prediction of iron-regulated genes under the control of the transcriptional regulatory protein Fur was done using computational analysis. The location of potential Fur box sequences was predicted using PredictRegulon server (http://180.149.48.104/predictregulon/index.html) using experimentally derived Fur box seed sequences from *N. gonorrhoeae*, *P. aeruginosa* and *E. coli* as input (Yellaboina *et al.*, 2004).

2.9.4.2. Expression of iron-regulated genes in *hbpA* mutant strain M601 and WT Manilae

2.9.4.2.1. Isolation of total RNA

Total cellular RNA was isolated from WT Manilae and *hbpA* mutant strain M601 using Trizol reagent. Briefly, 100 mL of WT and M601 culture grown in EMJH-BSA media (a) supplemented with 10 µg Fe mL^{-1} (HI) (b) preincubated with 400 µM EDDA (LI) (c) preincubated with 200 µM EDDA, containing 1 µM hemin as sole source of iron were harvested on day 7 by centrifugation at 12,000 x *g* for 10 min at 4°C and the cell pellets were resuspended thoroughly in 1 mL of Trizol reagent. 200 µL of chloroform was then added to the tubes which were mixed vigorously for 15 s followed by incubation at RT for 2 min. The tubes were centrifuged at 12,000 x *g* for 15 min at 4°C and the upper aqueous phase was transferred into a fresh tube. RNA was precipitated by the addition of equal volume of isopropanol, mixed and incubated at RT for 10 min. The tubes were then subjected to centrifugation at 12,000 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol, air dried and dissolved in 100 µL of RNase free water. The dissolved RNA was subjected to clean-up using RNeasy Mini kit (Qiagen, Netherlands) as per manufacturer's instructions. The samples were then treated with DNase I using TURBO DNA-free kit (Life technologies, USA) to remove any contaminating chromosomal DNA. The absence of genomic DNA was ensured by PCR (section 2.8.5.3.4) using $hbpA_{1449}$ primer set (FP; 5' –

GGGAATTCCATATGGAATTCAATACCACAGCCAACATGGG – 3' and RP; 5' - CCCAAGCTTTTAAAACTGGGCCGAGAATC) (Asuthkar *et al.*, 2007).

The RNA integrity was confirmed by agarose gel electrophoresis, followed by quantification using NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, DE, USA). The purified total RNA was stored at -80°C until further use.

2.9.4.2.2. qRT-PCR of WT and *hbpA* mutant strain M601 : determination of transcript levels of selected iron-regulated genes

The influence of iron limitation and presence of hemin on the expression of putative Fur-dependent genes (LA3468, LA2690, LB186, LA2242), Fur genes (LB183, LA1857, LA2887 and LA3094), TonB dependent receptor LA3149 and neighbouring genes of *hbpA* (LB192 and LB194) in wild-type Manilae and *hbpA* mutant strain M601 was studied by analyzing the transcript levels of the genes by qRT-PCR.

SuperScript II Reverse Transcriptase kit (Life technologies, USA) was used to convert 2 µg of RNA into cDNA following the manufacturer's instructions using the random primers supplied with the kit. The cDNA preparations were then used as templates in real time RT-PCR analysis (Pfaffl, 2001) to evaluate the relative levels of gene-specific mRNA transcripts in the total cellular RNA preparations. Gene-specific oligonucleotide primers (Table 2.10) were designed to produce products of approximately 70-150 bp. Primer binding efficiencies for these primer sets were determined using a standard curve. The cDNA preparations were used in reaction mixture containing 4 µL of cDNA (diluted 1:10), 5 pmol of each primer and 5 µL of 2X SYBR Green (Life Technologies, USA) in a total volume of 10 µL. PCR was performed in ABI 7500 Fast Sequence Detection System (Applied Biosystems, CA, USA). The amplification was done using the following program: 95°C for 5 min (melting) followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min (amplification). A melting curve analysis was performed after amplification to ensure the formation of a single product from the primer sets. Samples were assayed three times in triplicate with a no-template control (includes all of the qRT-PCR reagents except the RNA template) serving as a negative control on each reaction plate evaluated. The differences in the relative levels of specific

transcripts present in the RNA preparations obtained under different experimental conditions were calculated using using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Table 2.10. Sequence-specific primers used in qRT-PCR

Gene	Sequence (5' to 3')
16S rRNA	For – CGGTAATCCACGCCCTAAAC
	Rev – TCACTCTTGCGAGCATAGTC
hbpA	For – CAGGACGAGTGGACCGTTTC
	Rev – CCATAACTCGCTCTCCAAAC
LB 192	For – GAAACTGCGGCTTCTTCTAC
	Rev – CGCTTGTAAGTTCGGATACG
LB 194	For – CGGACGAAACGTTGCTGATT
	Rev – CTAGAATCGGCAAGTGGGCA
hemO	For – AATTATCGTACCGCGTTAGATTCC
	Rev – GGCAAGAATGGATTGTTTTTCG
LB 187	For – TCGCTCAATCTACGTCTTCG
	Rev – ATCTTTCCGGTTTCGGAAGG
LA 2690	For – ACGCCGACGAAGTGATTGAT
	Rev – AACCGCGATTACAATTCCGC
LB 183	For – TGAAATCCATCTTCCGGGAG
	Rev – GATGGTGATGATGACGGCTTG
LA 1857	For – CTCACCGCCGATCAGGTTT
	Rev – GCACGAGAAGCGTTTGGC
LA 2887	For – TTTGCCTTACTCCGGTGGAA
	Rev – GAATTGTGGGTTGTATAGAAACCGA
LA 3094	For – TTCAAGAACAAGCAGCAAGAGAA
	Rev – TCCGTAAATGTTCAAACTATGACCTG

LA 3468	For – TACCGGAGTTGCTTCCTCTGA
	Rev – TGACTTCGTGATCTCTTACGTTTCC
LA 2242	For – CCTTCCGTTGGTTTGATTGT
	Rev – CGCTTGAGAACCCCTATGAA
LA 3149	For – GAGGCCCGGGTAGTTTACTC
	Rev – CCGGAAAGAGGTGCTTTGTC

2.9.5. Characterization of LA3149

2.9.5.1. *In-silico* analysis of LA3149

Genomic sequences of LA3149 from *Leptospira spp.* and hemin/hemoglobin receptors of other pathogenic bacteria were accessed at the National Center for Biotechnology Information website (NCBI, MD, USA) (http://www.ncbi.nlm.nih.gov). BLAST was employed for all database searches (Altschul *et al.*, 1990) and primary amino acid sequence alignment was performed using the T-Coffee alignment algorithm (Notredame *et al.*, 2000) (http://www.igs.cnrs-mrs.fr/Tcoffee/). Signal peptide cleavage sites were predicted using the SignalP 3.0 server (Bendtsen *et al.*, 2004), which is accessible at http://www.cbs.dtu.dk/services/SignalP/. The presence of conserved domains were predicted using NCBI Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2011). The phylogenetic tree was drawn using MEGA 4.0 software (Tamura *et al.*, 2007) utilising the neighbour-joining method. The Protein Homology / Analogy Recognition Engine (Phyre) program (Bennett-Lovsey *et al.*, 2008) was used to predict three-dimensional structure of the protein. (http://www.sbg.bio.ic.ac.uk/phyre/).

2.9.5.2. Cloning and expression of LA3149

The 1062 bp encoding the C-terminal region (containing the FRAP-NNHL motif) of LA3149 gene was cloned as per protocol mentioned in section 2.8.5.3.1. Briefly, PCR amplification of the gene was done using genomic DNA of *L. interrogans* serovar Lai with primers (For; 5' - CCCGGATCCGCTAGACACCACT-3' and Rev; 5' - CCCCAAGCTTTGCTGTGAACGGAA-3') containing BamHI and HindIII

restriction sites, respectively (underlined bases in primer sequence). The 1062 bp amplicon was cloned into pET-22b (+) expression vector. The recombinant clone was selected on ampicillin (60 µg / mL) plate. The recombinant plasmids were confirmed by double digestion and a positive recombinant plasmid was transformed into the expression host *E. coli* BL21 (DE3). Expression of LA3149 was done following the same protocol mentioned in Section 2.8.5.3.9

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

Preparation of solutions

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

200 µL of hemin-agarose was washed thrice with 1 mL of wash buffer, with centrifugation done at 750 x g for 5 min. The hemin-agarose beads were incubated with 30 µg of uninduced and induced whole cell sonicates of *E. coli* BL21 (DE3) expressing the LA3149 protein for 60 min at 37ºC with gentle mixing. Agarose beads were then separated by centrifugation at 2,000 rpm for 10 min, and unbound proteins were removed by extensive washing with the following buffers; thrice with TNE buffer and twice with TN buffer. The beads were then suspended in 40 µL of sample buffer (2X) containing β-mercaptoethanol (1% v/v) and heated at 100ºC for 5 min and centrifuged for about 10 min. The supernatant was analyzed by 10% SDS-PAGE

CHAPTER III

RESULTS

3.1. Evaluation of the diagnostic potential of $HbpA_{55}$ - screening of serum samples for anti-HbpA antibodies by HbpA-ELISA

3.1.1. Preparation of pure $rHbpA_{55}$ antigen specific for *Leptospira* spp.

The $rHbpA_{55}$ protein, expressed by the 1449 bp *hbpA* clone, available in the lab was expressed as insoluble protein in the cell pellet (lane 2, Panel a) upon induction with 1 mM IPTG for 3 h at 37ºC. The inclusion bodies, containing the recombinant protein was subjected to Ni-NTA His-bind resin affinity chromatography under denaturing conditions. The eluates, containing the pure protein (lanes 4-10, Panel b) were pooled, concentrated and demonstrated to be pure by SDS-PAGE and immunoblotting (lanes 1 & 1', Panel C).

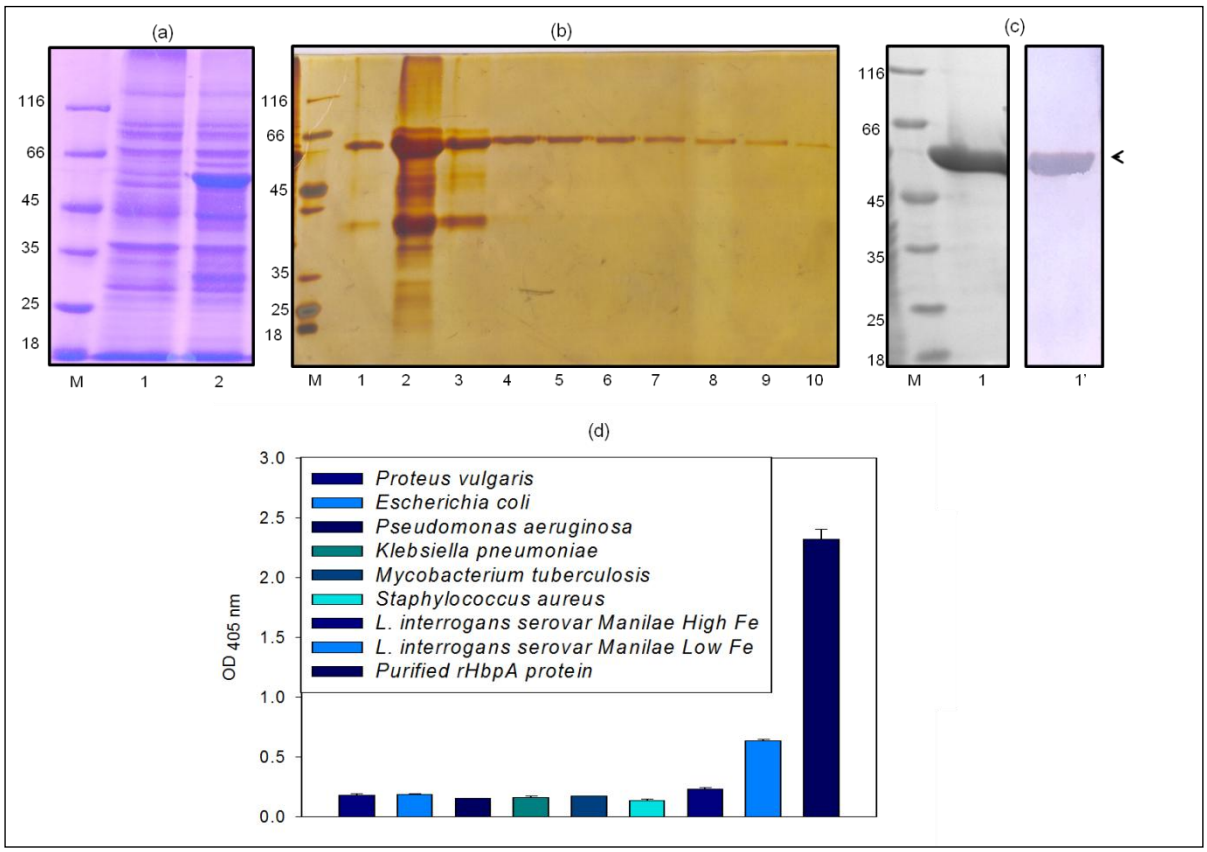


Fig. 3.1 Purification of $rHbpA_{55}$ and cross-reactivity of anti-HbpA antibodies with other bacterial proteins. Panel (a) shows the expression of $rHbpA_{55}$ in the insoluble pellet upon induction of the recombinant clone with IPTG (lane 2); lane 1 represent the uninduced sample. Panel (b) shows the purification of $HbpA_{55}$ by Ni-NTA column chromatography; lanes 1 & 2 represent the whole cell sonicates of the uninduced and

induced cell pellets of the clone, lane 3 is the flow through and the lanes 4-10 are the eluted fractions after washing the column. The purified protein was checked by SDS-PAGE (lane 1, Panel c) and by immunoblotting with anti-HbpA antibodies (lane 1'). Lane M in all the panels represents the protein molecular weight marker. The arrow indicates $rHbpA_{55}$. Panel (d) represents the titre obtained in ELISA done with rabbit anti-HbpA antibodies; the antigens included whole cell sonicates of several Gram-positive, Gram-negative bacteria and high and low iron *L. interrogans* serovar Manilae; $rHbpA_{55}$ was included as positive control. Error bars represent the standard deviation of the mean from two independent experiments set up in duplicate.

ELISA performed with whole cell sonicates of several Gram-positive and Gram-negative bacteria showed that rabbit anti-$HbpA_{55}$ antibodies reacted only with whole cell sonicates of *L. interrogans* serovar Manilae and did not react with other bacterial proteins (Fig. 1, Panel d). Purified $rHbpA_{55}$ was used as positive control and low iron serovar Manilae showed a three-fold increase in $OD_{405\ nm}$ when compared to high iron organisms, indicating the iron-regulated expression of the protein.

3.1.2. Serodiagnosis of leptospiral uveitis: performance of HbpA-ELISA *vs* MAT and commercial PanBio ELISA

In this study, serum from five groups (see Methods) were screened by ELISA for anti-$HbpA_{55}$ antibodies and compared with MAT and PanBio IgM ELISA kit for the diagnosis of leptospiral uveitis. MAT was done with all the samples in the five groups (Table 3.1). Groups III, IV and V representing non-leptospiral uveitis, cataract and endemic normals were tested negative by MAT. In Group I, of the 60 serum samples from patients clinically diagnosed with leptospiral uveitis, 30 were positive by MAT with titers ranging from 100 to 800; some of them showed mixed infection showing positivity with more than one serovar. The most predominant serogroups were Autumnalis (23.33%), Australis (20%), Icterohaemorrhagiae (16.66%) and Pyrogenes (16.66%) (Table 3.1). The remaining 30 patients tested negative by MAT. In Group II, comprising of systemic leptospirosis, MAT identified Icterohaemorrhagiae as the predominant serovar (Table 3.1).

ELISA-based identification of leptospiral uveitis is presented in Table 3.2. It shows the findings of PanBio ELISA and HbpA-ELISA in patients with leptospiral

uveitis (Group I) as compared to the other groups. In the latter, the cut-off value was calculated as 0.376 (Mean OD $_{405\ nm}$ of endemic normals + 2 x SD). The serum of patients with clinical signs of leptospiral uveitis (Group I), irrespective of MAT positivity showed significantly high levels of anti-HbpA antibodies as compared to controls ($P < 0.05$) (Fig. 3.2, Table 3.2). While the majority of samples in the control groups (Group III, IV and V) were negative, 2 of the non-leptospiral uveitis samples (Group III) and 3 of the cataract controls (Group IV) presented with titres higher than the cut-off value. Both the tests performed well in Group II samples (Table 3.2; $P < 0.05$ when compared to Group III, IV and V controls).

Table 3.1 MAT analysis of serum samples for the serodiagnosis of leptospiral uveitis

Group / MAT positivity	I (leptospiral uveitis)	II (systemic leptospirosis)	III (Non-leptospiral uveitis)	IV (Cataract controls)	V (Endemic normals)
Positive	30	20	none	none	none
Negative	30	-			
Serogroups identified by MAT					
Reactive serogroup	**Positive cases (n)**				
	Group I	**Group II**			
Australis	6	3			
Autumnalis	7	3			
Icterohaemorrhagiae	7	6			
Louisiana	2	1			
Semaranga	-	3			
Sejroe	2	-			
Pomona	1	-			
Pyrogenes	5	2			
Javanica	-	1			
Andamana	-	1			

It is evident that a large number of the leptospiral uveitis cases (Group I) were positive by the ELISA-based methods, with Table 3.3 showing a better correlation of both PanBio and HbpA-ELISA with clinical diagnosis as compared to

Table 3.2 Comparison of HbpA-IgG ELISA and PanBio IgM ELISA in the sero diagnosis of leptospiral uveitis

	Group I		Group			
	MAT positive	MAT negative	II	III	IV	V
HbpA- IgG ELISA	28/30 (93)	27/30 (90)	17/20 (85)	3/20 (15)	3/10 (30)	1/20 (5)
PanBio IgM ELISA	27/30 (90)	22/30 (73)	8/20 (40)	8/20 (40)	4/10 (40)	3/20 (15)

The values represent the numbers tested positive / total number of samples tested; % positivity is given in parentheses.

Table 3.3 Higher correlation of PanBio and HbpA-ELISA with clinical diagnosis in Group I cases of leptospiral uveitis

a) Correlation of the total number of cases

	MAT			Clinical diagnosis		
	+	-	Total	+	-	Total
HbpA-IgG ELISA						
+	28	34	62	55	7	62
-	2	46	48	5	43	48
Total	30	80	110	60	50	110
Pan Bio IgM-ELISA						
+	27	37	64	49	15	64
-	3	43	46	11	35	46
Total	30	80	110	60	50	110

b) Sensitivity and specificity of the two ELISA-based methods with MAT and clinical diagnosis

	MAT		Clinical diagnosis	
	HbpA-IgG	Pan Bio	HbpA-IgG	Pan Bio

	ELISA	IgM-ELISA	ELISA	IgM-ELISA
Sensitivity (%)	93	90	92	82
Specificity (%)	58	54	86	70

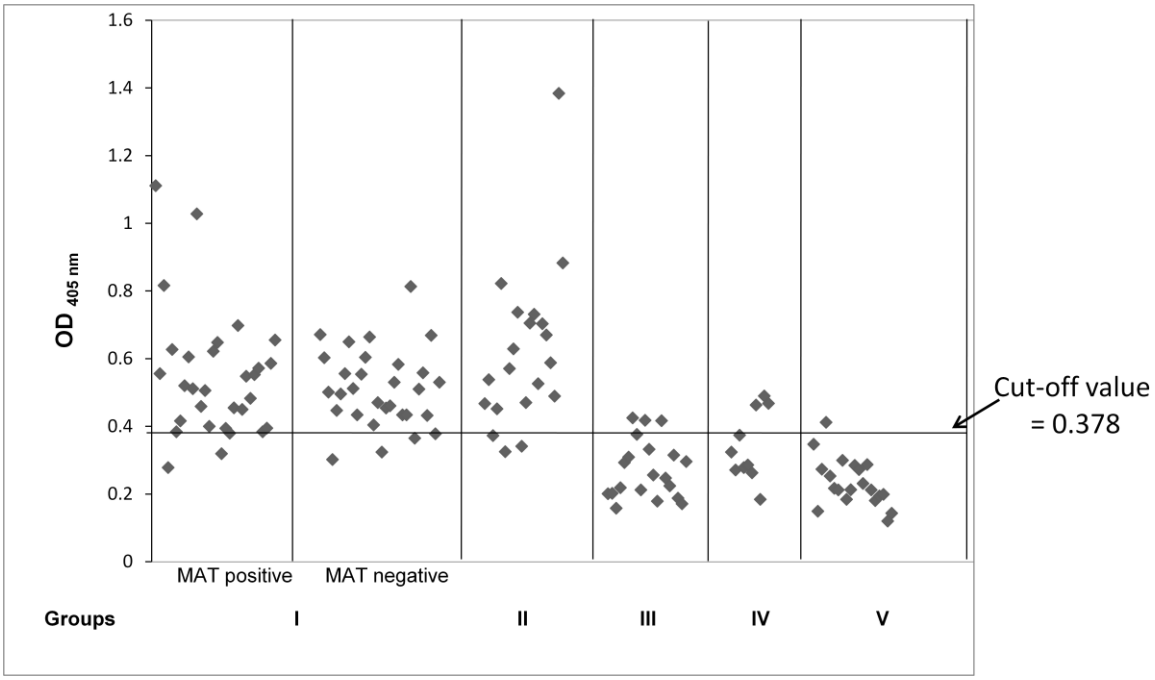


Fig 3.2 Levels of anti-HbpA antibodies in the serum of patients with leptospiral uveitis as determined by ELISA. The figure represents the titre of anti-HbpA antibodies in the serum of patients with leptospiral uveitis (Group I), systemic leptospirosis (Group II), non-leptospiral uveitis (Group III), cataract cases (Group IV) and normal healthy controls (Group V). The non-parametric Kruskal-Wallis test showed significant differences between patient and control groups (P<0.05). The cut-off value is indicated by the arrow.

MAT. While sensitivity with MAT as the reference test was higher, the specificity was low that can be attributed to the lower number of serovars included in the assay (discussed in detail in Chapter IV).

3.1.3 IgG- *vs* IgM-HbpA-ELISA in the screening of serum samples from patients with systemic leptospirosis

35 MAT positive serum samples from human subjects clinically diagnosed with systemic leptospirosis (Group VI) were screened for both IgG - and IgM-specific anti-HbpA antibodies. The predominant serogroup determined by MAT was Icterohaemorrhagiae (57%); others included Grippothyphosa (17%), Australis (5%)

with one positive each for Pyrogenes, Canicola, Pomona, Louisiana, Ranarum, Hebdomadis and Autumnalis serovars. The 15 endemic normals, included in the test were negative by MAT.

Fig. 3.3 shows the identical performance of both IgG and IgM-based HbpA-ELISA. Both the antibody levels were higher than the control that was found to be statistically significant ($P < 0.05$). Table 3.4 lists the number of cases identified positive by HbpA-ELISA (IgM and IgG, Panels a & b) and PanBio ELISA (Panel c) when compared to MAT. The sensitivity and specificity of these tests are shown in Panel d.

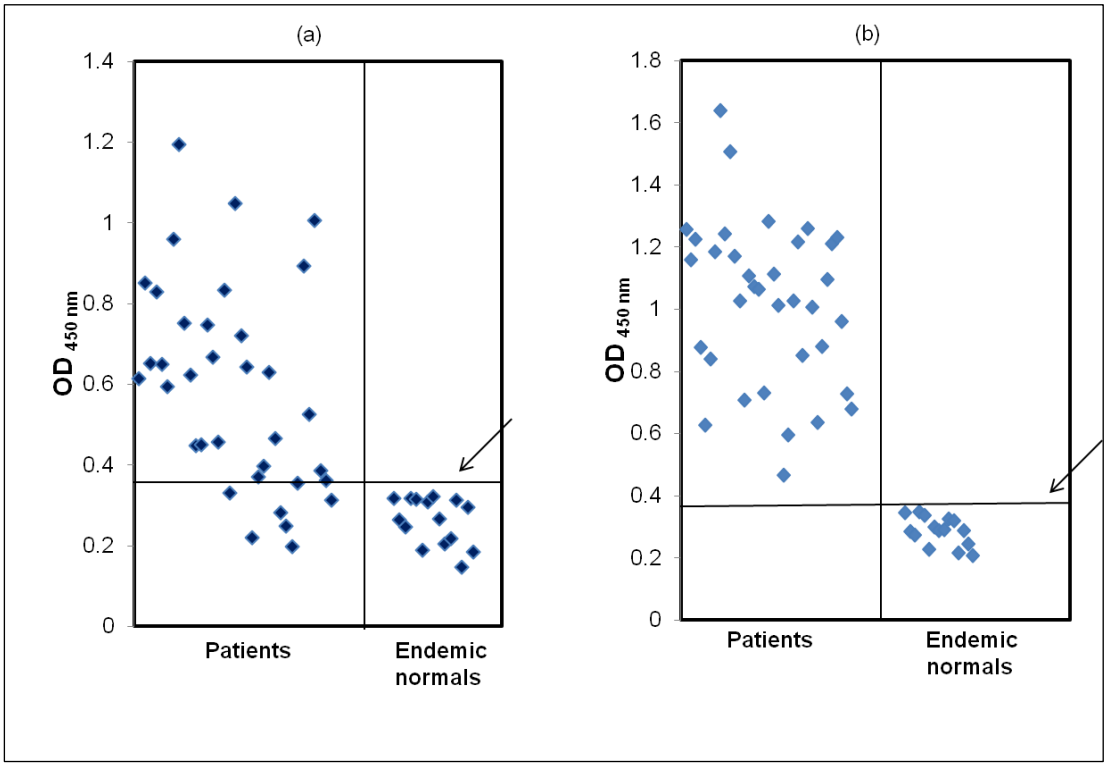


Fig. 3.3 IgM & IgG-specific anti-HbpA antibodies in the serum of patients with systemic leptospirosis. ELISA was done with rHbpA$_{55}$ as antigen, using patient's serum at a dilution of 1:200. Panel (a) & (b) show the level of IgM and IgG anti-HbpA antibodies in the patient's samples and the endemic controls. The non-parametric Kruskal-Wallis test showed significant differences between patient and control group (P<0.05). The arrows indicate the cut-off determined from the titres obtained with serum from endemic normals.

Table 3.4 Performance IgG and IgM-based HbpA-ELISA as compared to MAT and PanBio IgM ELISA for the serodiagnosis of systemic leptospirosis

(a) IgM-specific HbpA-ELISA *vs* MAT

		MAT		Total
		Positive	Negative	
IgM-specific HbpA ELISA	Positive	26	0	26
	Negative	9	15	24
Total		35	15	50

(b) IgG-specific HbpA-ELISA *vs* MAT

		MAT		Total
		Positive	Negative	
IgG-specific HbpA ELISA	Positive	35	0	35
	Negative	0	15	15
Total		35	15	50

(c) PanBio ELISA *vs* MAT

		MAT		Total
		Positive	Negative	
PanBio IgM ELISA	Positive	31	4	35
	Negative	4	11	15
Total		35	15	50

d) Sensitivity and specificity of ELISA *vs* MAT

Parameter	**MAT**		
	HbpA-IgM ELISA	**HbpA-IgG ELISA**	**Pan Bio IgM-ELISA**
Sensitivity (95% CI)	74.29 (56.74-87.48)	100 (89.90-100)	88.57 (73.24-96.73)
Specificity (95% CI)	100 (78.03-100)	100 (78.03-100)	73.33 (44.9-92.05)
Positive predictive value (95% CI)	100 (86.65-100)	100 (89.90-100)	88.57 (73.24-96.73)
Negative predictive value (95% CI)	62.50 (40.60-81.16)	100 (78.03-100)	73.33 (44.91-92.05)

3.1.4. HbpA-ELISA *vs* MAT and LipL32 / LipL41 ELISA in the screening for bovine leptospirosis

A total of 176 bovine serum samples was tested by MAT and ELISA using HbpA, LipL32 and LipL41 as antigens. 23% of the samples (41) of the samples were positive by MAT; Canicola was the reactive serovar in 46% of the positive samples, with the others including Pomona (19.51%), Hardjo (19.51%), Lai (12.19%) and Tarassovi (2.4%).

ELISA done with all the three leptospiral-specific antigens showed the superior performance of ELISA as compared to MAT (Table 3.5).

Table 3.5 Comparison of ELISA with MAT in the screening of bovine samples for leptospirosis

(a) HbpA-ELISA *vs* MAT

		MAT		Total
		Positive	Negative	
HbpA	Positive	41	50	91
	Negative	0	85	85
Total		41	135	176

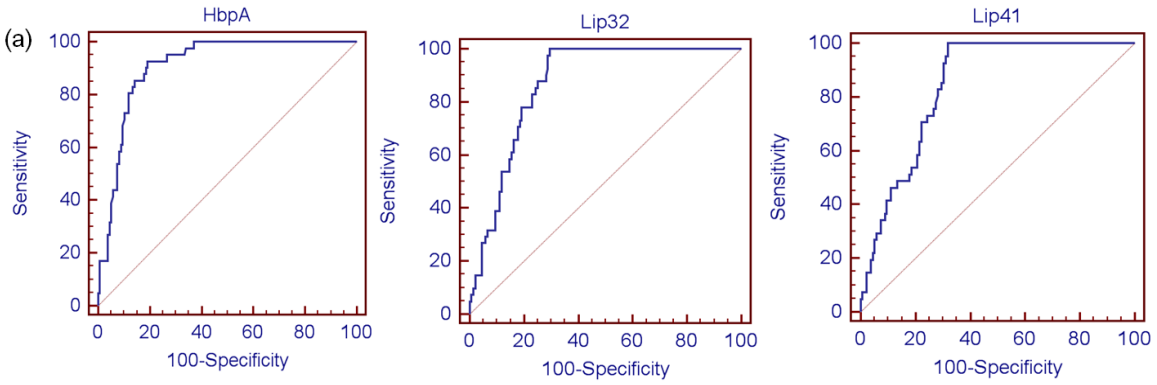
(b) LipL32-ELISA *vs* MAT

		MAT		Total
		Positive	Negative	
LipL32	Positive	41	42	83
	Negative	0	93	93
Total		41	135	176

(c) **LipL41-ELISA *vs* MAT**

		MAT		Total
		Positive	Negative	
LipL41	Positive	41	47	88
	Negative	0	88	88
Total		41	135	176

There was good concordance of the three antigens as evident from the ROC curves for the three antigens (Fig. 3.4, Panel a). The data generated by statistical analysis (Panel b) showed 100% sensitivity of all the three antigen-based ELISA tests with MAT. As also observed earlier, the specificity was low (≥ 63%). The table gives the cut-off values for each of the antigens and all values above this cut-off can be considered as positive. The % positivity, determined to be 51.7, 50 and 47.15 for HbpA, LipL41 and LipL32 respectively, were higher than MAT (23%). From Table 3.6, the κ values of 0.920 & 0.887 for HbpA-ELISA vs LipL41-ELISA & LipL32 shows the high degree of agreement among the three antigen-based ELISA tests as evaluated by Kappa statistical analysis.



(b) ROC curve analysis of ELISA *vs* MAT

Parameter	Antigens used in ELISA		
	HbpA	LipL32	LipL41
AUC (95% CL)*	0.909(0.856-0.947)	0.866(0.807-0.913)	0.84(0.779-0.892)
Standard error	0.032	0.037	0.040
Cut-off (%)	27.94	23.51	32.28
% Sensitivity (95% CL)*	100 (91.4-100.0)	100 (91.4-100.0)	100 (91.4-100.0)
% Specificity (95% CL)*	62.96 (54.2-71.1)	68.89(60.4-76.6)	65.19 (56.5-73.2)
Positive predictive value (%)	45.05	49.39	46.59
Negative predictive value (%)	100	100	100

Fig. 3.4 ROC curve analysis of ELISA (HbpA / LipL32 / LipL41) *vs* MAT. Panel (a) shows the 'Receiver Operating Characteristic' **(**ROC) curves generated from MAT and ELISA done with HbpA, LipL32 and LipL41 as antigens. Using MedCalc software (Version 11.5.0.0), the curves were generated by plotting sensitivity against 100-specificity to

determine the optimal cut-off for ELISA. Panel (b) shows the data generated by statistical analysis of the ROC curves; * represents 95% confidence limits

Table 3.6 κ statistics: high degree of agreement among the three antigen-based ELISA tests

		LipL41		LipL32	
		Positive	Negative	Positive	Negative
HbpA	Positive	86	5	81	10
	Negative	2	83	1	84

Serological test	κ value (95% CI)
HbpA vs LipL32 ELISA	0.887 (0.819- 0.955)
HbpA vs LipL41 ELISA	0.920 (0.863- 0.978)

3.2. Generation of monoclonal antibodies against HbpA of *L. interrogans* serovar Lai and evaluation of their potential in antigen detection in serum samples

3.2.1. Development of monoclonal antibodies against $HbpA_{55}$ protein

3.2.1.1. Immunisation of mice with $rHbpA_{55}$ and determination of the titre of polyclonal anti-HbpA antibodies in serum samples

All the four mice immunised with $rHbpA_{55}$ produced antibodies against the antigen, as evident from the immunoblot analysis (Fig. 3.5). However, in two mice, increasing titre of anti-HbpA antibodies (Fig. 3.6; $OD_{450\ nm}$ of 1.959 and 1.883 in mice 1 & 2 respectively), with negligible cross-reactivity with *E. coli* proteins (A_{450nm} < 0.093) led us to choose the spleens from these two mice for fusion to generate the hybridomas.

3.2.1.2. Production and screening of hybridomas

Hybridomas were generated upon fusion of the sensitised spleen lympho cytes with myeloma cells, as described in Methods. A large number of hybridomas were generated from the two spleen samples, as listed in Table 3.7. As indicated, 54

clones producing high titers of anti-HbpA antibodies were chosen for further screening.

Of the 54 hybridomas, 10 polyclones (1F3.PC, 2C3.PC, 3G9.PC, 5C5.PC, 6D10.PC, 8B3.PC, 10G7.PC, 11B5.PC, 13C6.PC, 14G3.PC) expressing high anti-HbpA antibodies were subjected to first limiting dilution cloning (Fig. 3.7).

Following first limiting cloning, 68 HbpA antibody producing clones were obtained of which 24 clones were selected and rescreened. Figure 3.8 shows the anti-HbpA antibody titers obtained on first limiting cloning from which 6 monoclones (2E5.1C, 8B9.1C, 10D7.1C, 1B9.1C, 4F2.1C and 5F3.1C); 3 additional polyclones (6F7.2C, 7B7.2C, 8C7.2C) secreting high titres of anti-HbpA antibodies were chosen for second limiting dilution cloning. From the latter, out of 101 monoclones, four clones (Fig. 3.9), namely 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C were chosen.

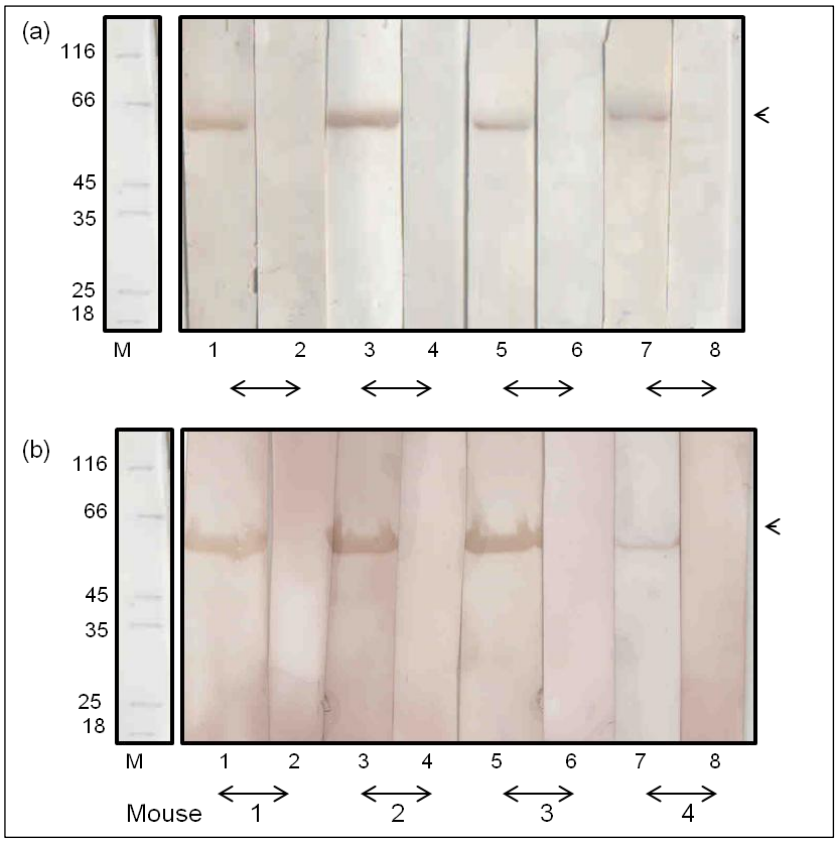


Fig. 3.5 Western blotting: detection of polyclonal anti-HbpA antibodies in Balb/c mice immunized with rHbpA$_{55}$. In both panels (a) & (b), 5 µg of rHbpA$_{55}$ (lanes 1, 3, 5 and 7) and 30 µg of cell lysate of *E. coli* BL21 (DE3) (lanes 2, 4, 6 and 8) were subjected to SDS-PAGE and immunoblotting with serum obtained from the four mice on days 14 (Panel a) & 21 (Panel b) after immunization. Serum from each mouse reacted with

rHbpA$_{55}$ and no cross-reacting bands against *E. coli* proteins were seen in both the panels. Lane M represents molecular weight marker.

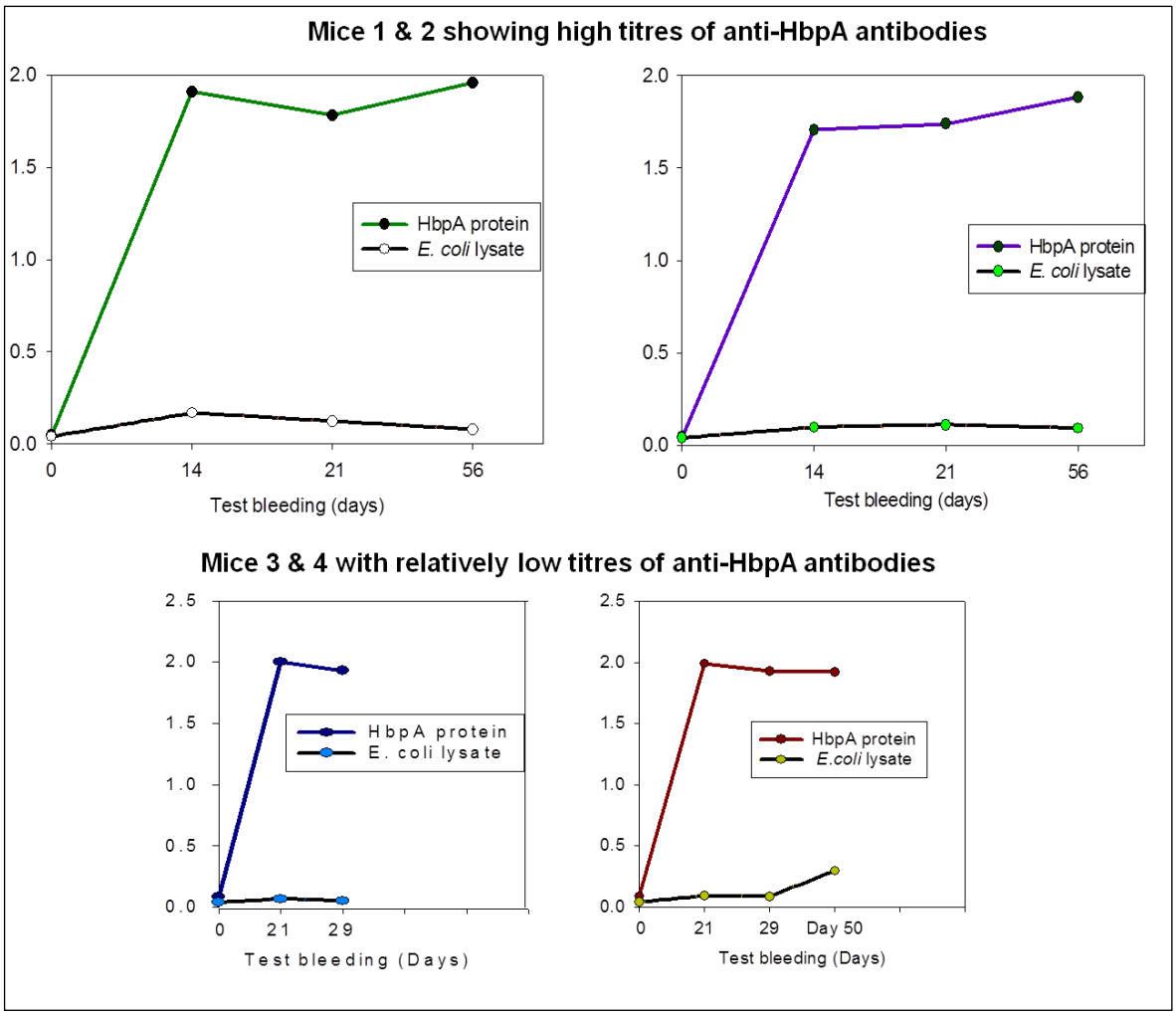


Fig. 3.6 Determination of the titre of anti-HbpA antibodies in the serum samples from the four immunized mice. The microtitre plate was coated with 250 ng of rHbpA$_{55}$ and 500 ng of *E. coli* lysate and reacted with serum from the four immunised mice on different days after immunisation, inclusive of the sample collected on day 0. The four panels show the antibody titre in the four mice.

Table 3.7 Screening and selection of hybridomas secreting anti-HbpA antibodies

Number of hybridomas	Mouse 1	Mouse 2
Total	462	462
Producing anti-HbpA antibodies	107	166
Selected for upscaling	22	32
	54	

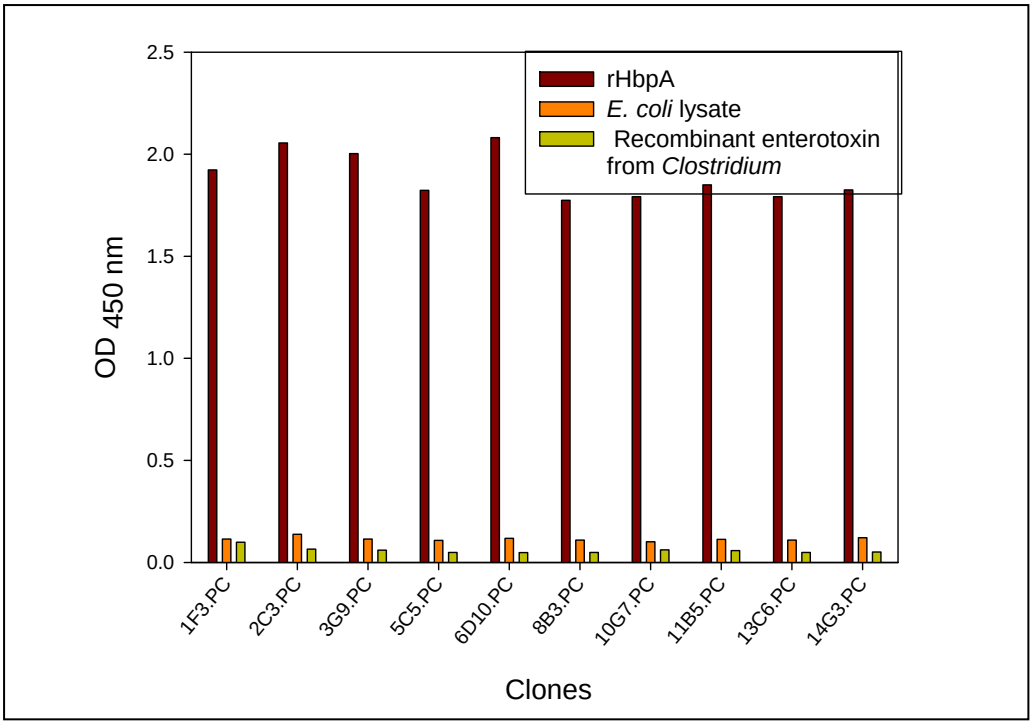


Fig. 3.7 Primary screening of hybridoma clones for anti-HbpA antibody production by ELISA. As indicated in Table 3.7, the supernatant of 54 clones were screened for anti-HbpA antibodies. The figure shows 10 polyclones reacting with $rHbpA_{55}$ as antigen. *E. coli* lysate and a His-tagged protein (recombinant enterotoxin protein from *Clostridium* spp.) were included as antigen to check for cross-reacting anti-*E. coli* antibodies and antibodies against the His-tag to ensure that such clones were excluded from the selection process.

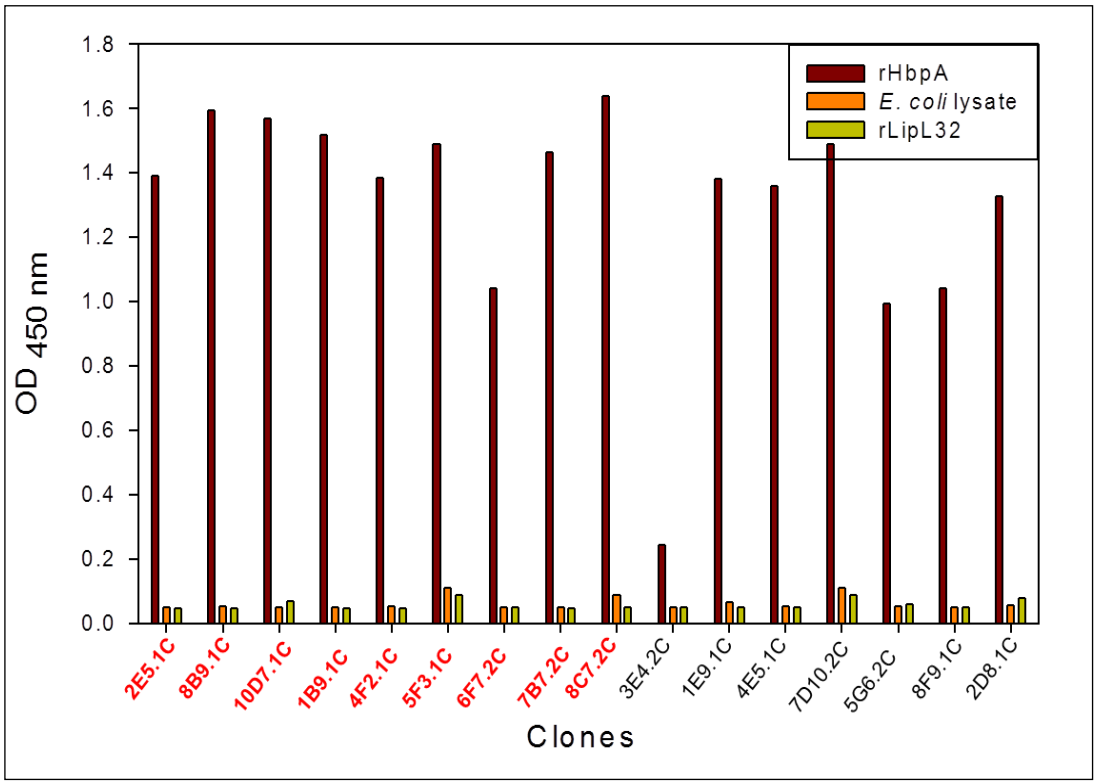


Fig. 3.8 Selection of clones for second limiting dilution. Supernatant from 68 clones obtained after 1st limiting dilution were screened for anti-HbpA antibodies. The figure shows 16 clones reacting with $rHbpA_{55}$ as antigen of which 3 polyclones and 6 monoclones bold-faced and highlighted in red were selected for 2nd limiting dilution. *E. coli* lysate and a His-tagged protein (rLipL32) were included as antigen to check for cross-reacting anti-*E. coli* antibodies and antibodies against the His tag to ensure that such clones were excluded from the selection process.

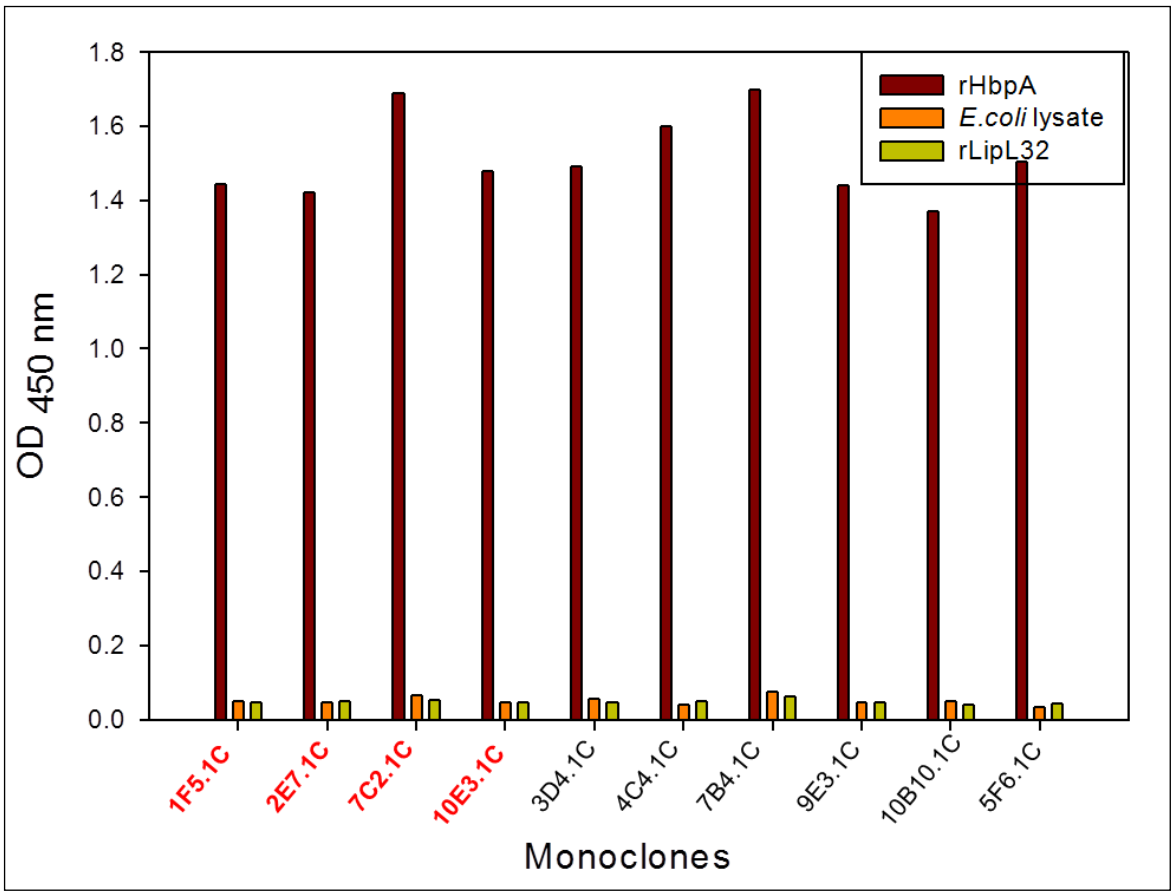


Fig. 3.9 Final selection of four clones Supernatant from 101 monoclones obtained after 2nd limiting dilution were screened for anti-HbpA antibodies. The figure shows 10 clones reacting with $rHbpA_{55}$ as antigen of which 4 monoclones 1F5.1C, 2E7.1C, 7C2.1C, 10E3.1C producing high titer of anti-$HbpA_{55}$ antibody were selected and upscaled for further characterisation. *E. coli* lysate and a His-tagged protein (rLipL32) were included as antigen to check for cross-reacting anti - *E. coli* antibodies and antibodies against the His tag to ensure that such clones were excluded from the selection process.

3.2.1.3. Characterisation of the monoclonal antibodies (MAbs) produced by the four clones

The four MAbs were tested for their isotypes and specificity for *Leptospira* spp. The former, done with the commercial Pierce Rapid ELISA mouse MAb isotyping kit is represented in Table 3.8.

Table 3.8 Isotypes of the four MAbs

MAbs / Isotype	1F5.1C	2E7.1C	7C2.1C	10E3.1C
Subclass	IgG_{2b}	IgG_{2b}	IgG_{2b}	IgG_{2b}
Light – chain	Kappa	Kappa	Kappa	Kappa

ELISA, done with the four MAbs showed negligible reactivity (Fig. 3.10) with bacterial lysates of commonly encountered pathogens including *P. vulgaris*, *P. aeruginosa*, *K. pneumoniae, E. coli, S. aureus* and *M. tuberculosis* (mean A_{450nm} < 0.150). All the MAbs showed reactivity with *L. interrogans* serovar Manilae; a higher titre with low iron organisms is due to the increased expression of HbpA under iron limiting conditions.

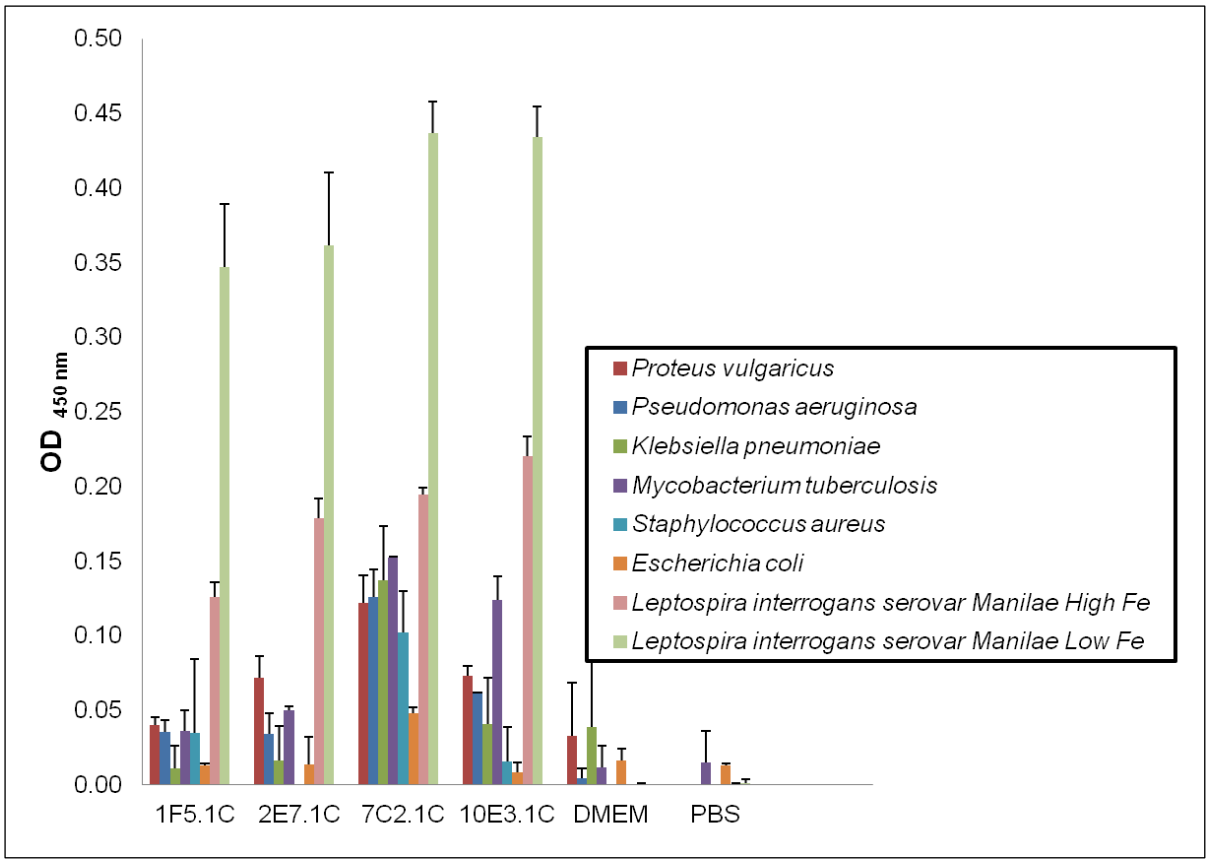


Fig. 3.10 Specificity of monoclonal antibodies to HbpA antigen The figure represents the titre obtained in ELISA done with 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C monoclonal antibodies; the antigens included whole cell sonicates of several Gram-positive, Gram-negative bacteria and high and low iron *L. interrogans* serovar Manilae; DMEM media and PBS were used as negative controls for the assay. Error bars represent the standard deviation of the mean from the experiment set up in triplicate.

3.2.2. Characterisation of the MAbs: recognition of antigenic determinants in HbpA

Three fragments spanning the full length HbpA were expressed as recombinant proteins and the specificity of each of the four MAbs for these antigenic fragments are presented below.

3.2.2.1. Cloning and expression of $HbpA_{20}$, $HbpA_{25}$ and $HbpA_{34}$

The three fragments *hbpA*$_{484}$, *hbpA*$_{566}$ and *hbpA*$_{871}$, extending over the full length *hbpA* (Fig. 3.11a & 3.11b) were cloned into pET28a (+) as detailed in Methods. The clones were confirmed by sequence analysis of the PCR amplified products. The three recombinant proteins $HbpA_{20}$, $HbpA_{25}$ and $HbpA_{34}$ were obtained as insoluble proteins upon induction with 1 mM IPTG for 3 h at 37°C (Fig. 3.11c). The inclusion bodies were solubilised and purified under denaturing conditions by Ni-NTA His-bind resin affinity chromatography.

3.2.2.2. Affinity-purified HbpA antigens: analysis by SDS-PAGE and immunoblotting

All the three recombinant proteins of approximate molecular mass 20, 25 and 34 kDa respectively were seen as single bands in SDS-PAGE (Fig. 3.12a) and in the immunoblot developed with anti-His antibodies (Fig. 3.12b). The $rHbpA_{55}$ was included as a control.

3.2.2.3. Immunoreactivity of the four MAbs with $HbpA_{20}$ / $HbpA_{25}$ / $HbpA_{34}$

Of the four MAbs, 7C2.1C and 10E3.1C were specific for $HbpA_{34}$ (Fig. 3.13) with the other two 1F5.1C and 2E7.1C reacting with all the three HbpA antigens, as observed by ELISA (Fig. 3.13) and by immunoblotting analysis (Fig. 3.14a). The observations are summarised in Fig. 3.14b.

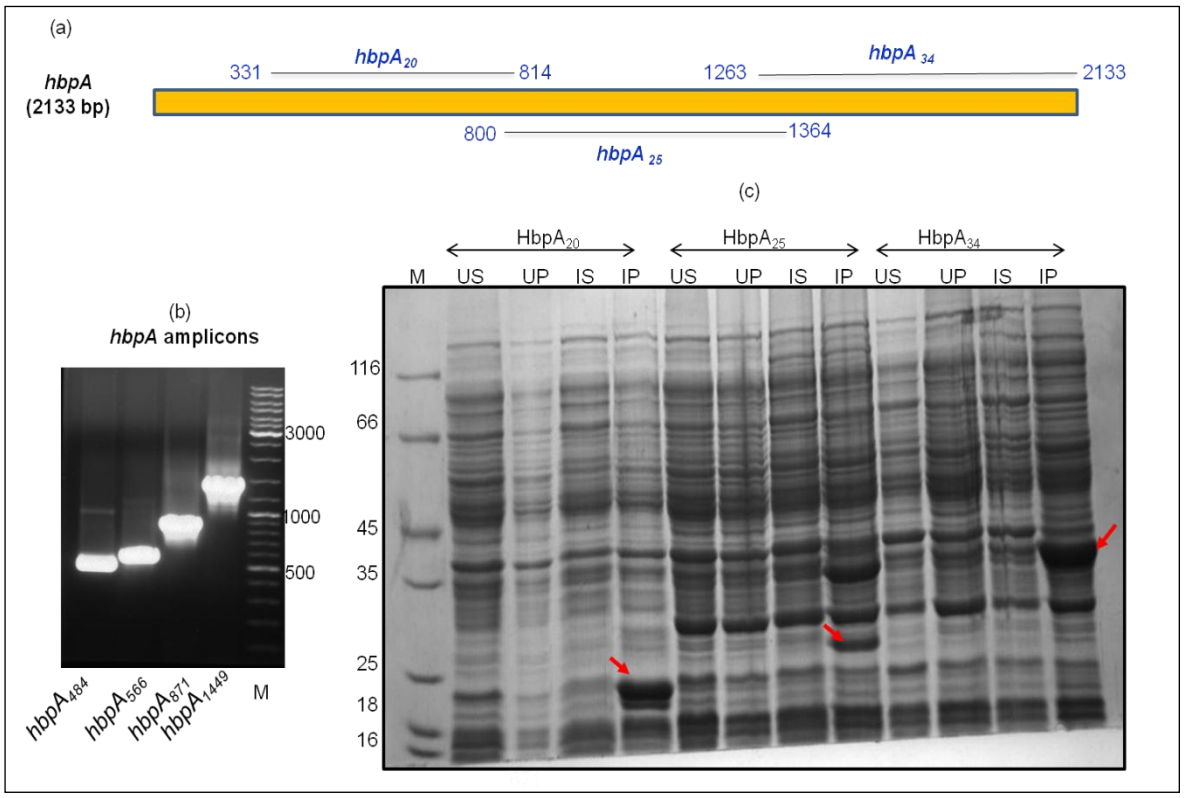


Fig. 3.11 Cloning and expression of $HbpA_{20}$, $HbpA_{25}$ & $HbpA_{34}$. Panel (a) shows the scheme for the amplification of the three *hbpA* fragments, with Panel (b) showing $hbpA_{484}$, $hbpA_{566}$ and $hbpA_{871}$. Panel (c) shows the SDS-PAGE separation of $rHbpA_{20}$, $HbpA_{25}$ and $HbpA_{34}$ in the respective cell pellets upon induction with IPTG. The uninduced supernatant and pellets of the respective recombinant clones are indicated as US & UP and the corresponding induced samples are indicated as IS and IP.

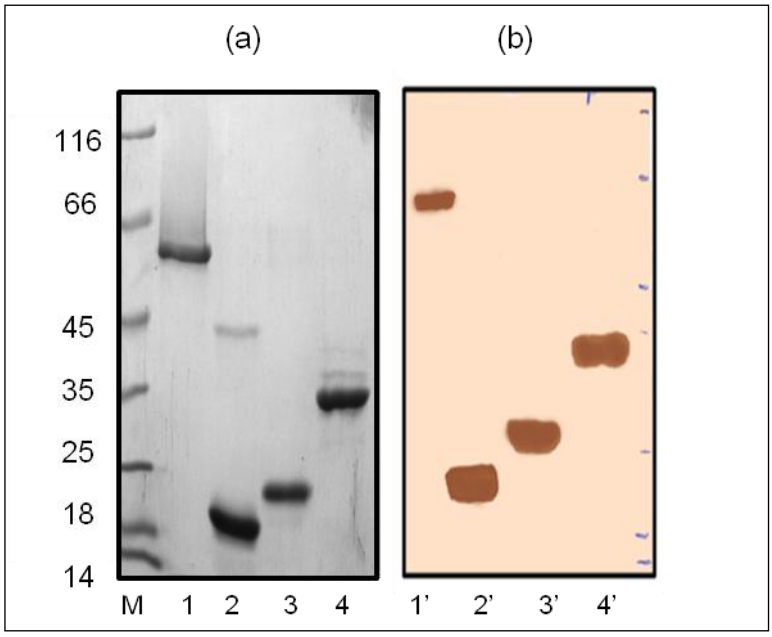


Fig. 3.12: Demonstration of the purity of $rHbpA_{20}$, $rHbpA_{25}$ and $rHbpA_{34}$. Panel (a) & (b) shows the SDS-PAGE and immunoblot analysis with anti-His antibodies of $rHbpA_{55}$ (lanes 1 &1'), $HbpA_{20}$ (lanes 2 & 2'), $HbpA_{25}$ (lanes 3 & 3') and $HbpA_{34}$ (lanes 4 & 4'), as purified by Ni-NTA column chromatography. Lane M represents the molecular weight marker.

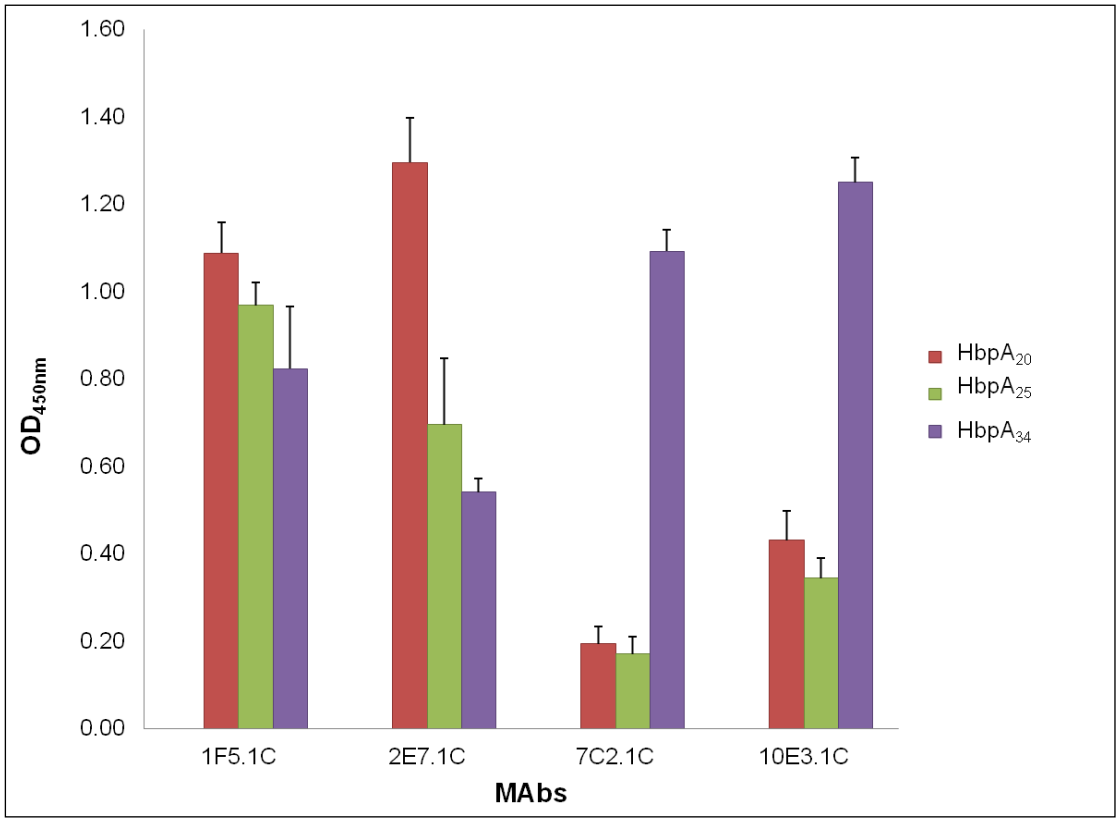
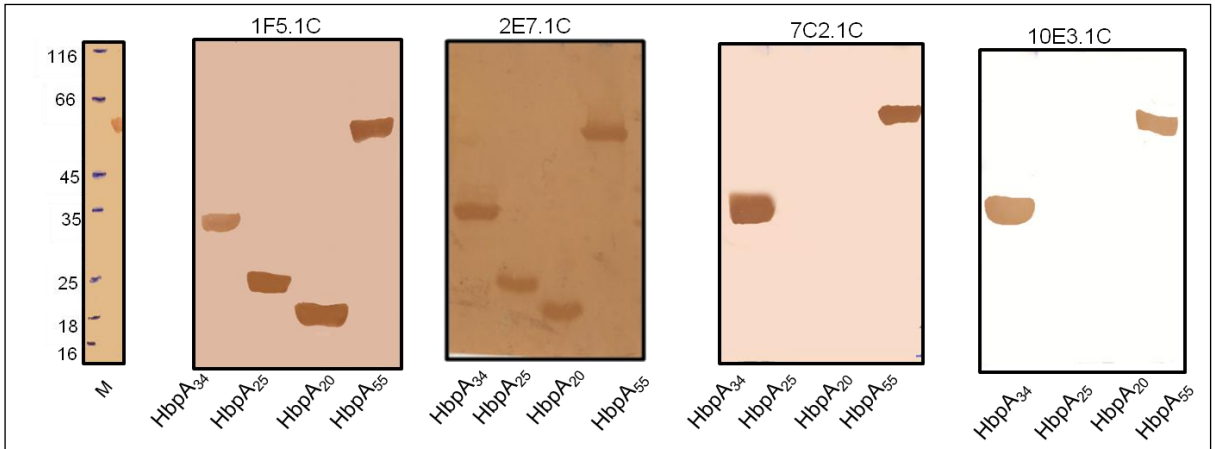


Fig. 3.13 ELISA: recognition of the HbpA antigens by the four MAbs. 250 ng of $HbpA_{20}$, $HbpA_{25}$ and $HbpA_{34}$ were reacted with MAbs 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C. HRP-conjugated goat anti-mouse IgG antibody was used as the secondary antibody-enzyme conjugate and the absorbance of the colour developed with TMB/H_2O_2 was read at 450 nm. The error bars represent the standard deviations of the mean of three identical experiments.

3.2.3. Evaluation of the antigen-detection potential of the four MAbs

All the four MAbs 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C were tested for their ability to detect HbpA in serum samples. As our serum samples were collected randomly, we first performed ELISA with fetal calf serum spiked with HbpA and then extended to clinical samples.



(b)

HbpA antigen	Immunoreactivity with: Anti-His Ab	Polyclonal anti-HbpA Ab	1F5.1C	2E7.1C	7C2.1C	10E3.1C
$HbpA_{20}$	•	•	•	•	-	-
$HbpA_{25}$	•	•	•	•	-	-
$HbpA_{34}$	•	•	•	•	•	•

Fig. 3.14 Immunoblot analysis: specificity of the MAbs for HbpA antigens. Panel (a) shows the immunoblots of MAbs 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C with $HbpA_{20}$, $HbpA_{25}$ and $HbpA_{34}$; $HbpA_{55}$ was included as control. Lane M represents protein molecular weight marker. Panel (b) summarizes the observations seen above and in Fig. 3.13

3.2.3.1. Antigen detection in fetal calf serum spiked with HbpA: determination of the lowest HbpA concentration by sandwich ELISA

Sandwich ELISA was performed by coating the microtitre plate with polyclonal rabbit anti-HbpA antibody, followed by addition of fetal calf serum to which was added $rHbpA_{55}$, ranging from 0 - 1000 ng (Fig. 3.15a). Four such identical plates were then incubated with MAb 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C respectively and the performance of these MAbs was found to be good. From Fig. 3.15a, it can be seen that 10E3.1C could detect as low as 0.48 ng of HbpA; the detection limit of 2E7.1C and 7C2.1C was ~ 2 ng while 1F5.1C was relatively less sensitive, with the detection limit being 4 ng. When whole cell sonicates of low iron serovar Manilae was used (Fig. 3.15b), the detection limit followed a similar trend as for $rHbpA_{55}$, with 10E3.1C reacting with as low as 62.5 ng and the other three MAbs showing a detection limit of 125 ng.

3.2.3.2. Use of the MAbs to detect HbpA in serum samples from patients with leptospirosis

Based on the performance of MAb 10E3.1C, it was used to detect HbpA in serum samples from human patients with systemic leptospirosis and leptospiral uveitis; samples with low MAT titers (1:100) were arbitrarily chosen for the assay. Sandwich ELISA (Fig. 3.16) showed that the mean A_{450nm} in these test samples, determined to be 0.283 ± 0.051 was only marginally higher than the healthy controls (mean A_{450nm} 0.122 ± 0.009). However, when the samples were spiked with 1 ng of rHbpA antigen, the increase in A_{450nm} reflected the addition of HbpA in the samples (A_{450nm} = 0.570 ± 0.068).

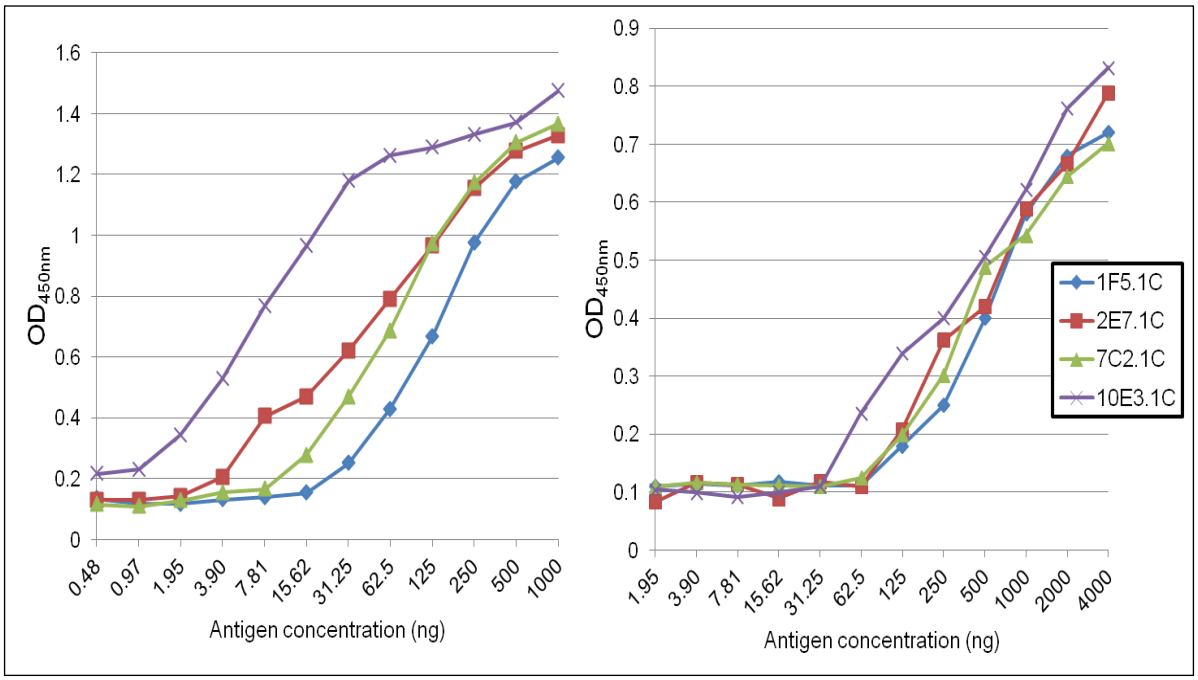


Fig. 3.15 Sandwich ELISA: antigen detection in fetal calf serum spiked with HbpA. Sandwich ELISA was performed as detailed in Methods. Panels (a) & (b) represent FCS spiked with $rHbpA_{55}$ and whole cell sonicate of low-iron grown serovar Manilae. ELISA was performed in triplicate and mean values are plotted.

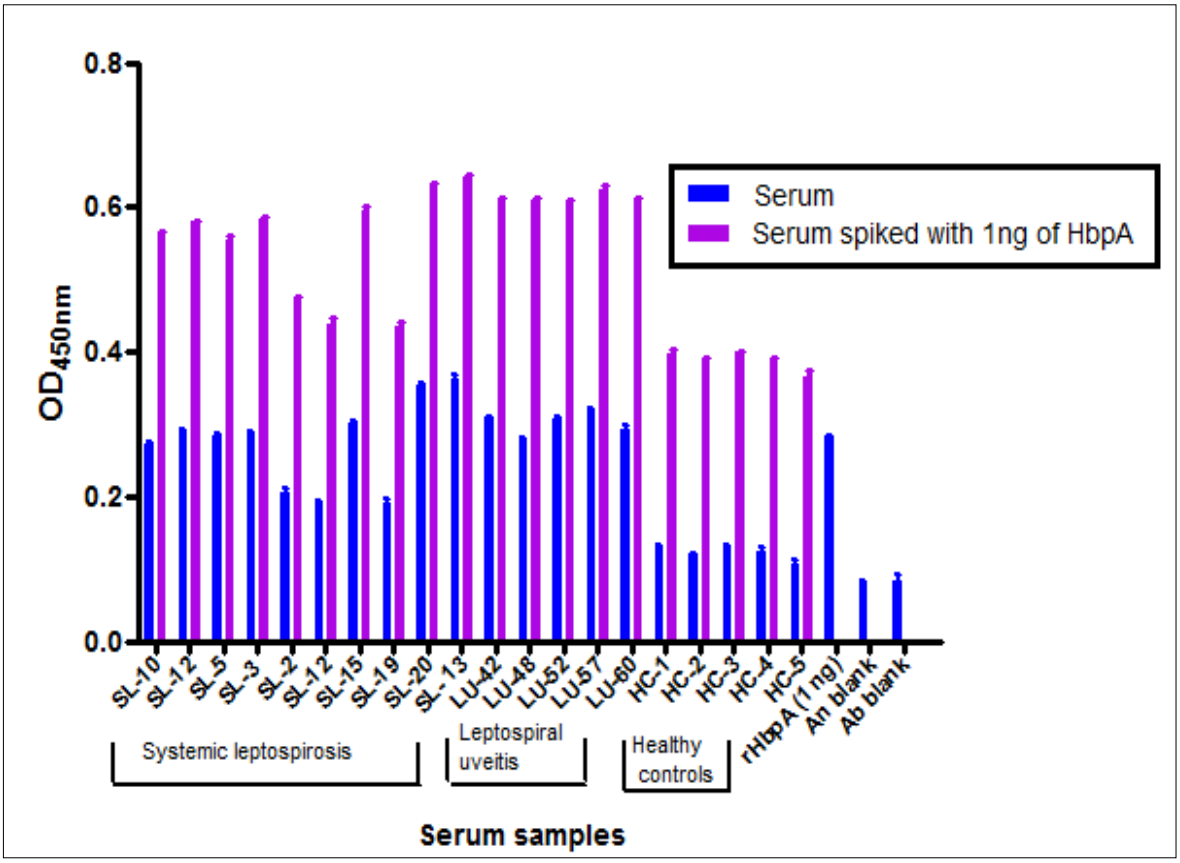


Fig. 3.16 Detection of HbpA in serum samples from patients suspected of leptospirosis. The test, done essentially as detailed in Methods, was performed in two identical sets, one with serum from patients with systemic leptospirosis and leptospiral uveitis added as such and in the other spiked with 1 ng of $rHbpA_{55}$. ELISA was performed in triplicate and mean values are plotted with error bars representing standard deviation.

3.3. Identification of a second hemin-binding protein LA3149 (HbpB) in *L. interrogans* serovar Manilae: studies with the *hbpA* mutant strain M601

3.3.1. *hbpA* mutant strain M601: insertional inactivation of *hbpA* confirmed by PCR and expression studies

hbpA mutant strain M601, generated by random transposon mutagenesis in *L. interrogans* serovar Manilae strain L495 (Murray *et al.*, 2009a) was a kind gift from Prof. Ben Adler, Monash University. In Figure 3.17(a), the chromosome II of the mutant strain M601 shows the insertional inactivation of *hbpA* (LB191) by the 2.2 kb kan^R - containing Himar1 transposon. The bigger transcript (Fig. 3.17b, lane 2) obtained by PCR of the genomic DNA of the mutant strain using *hbpA*-specific primers confirmed the presence of the 2.2 kb transposon within *hbpA*.

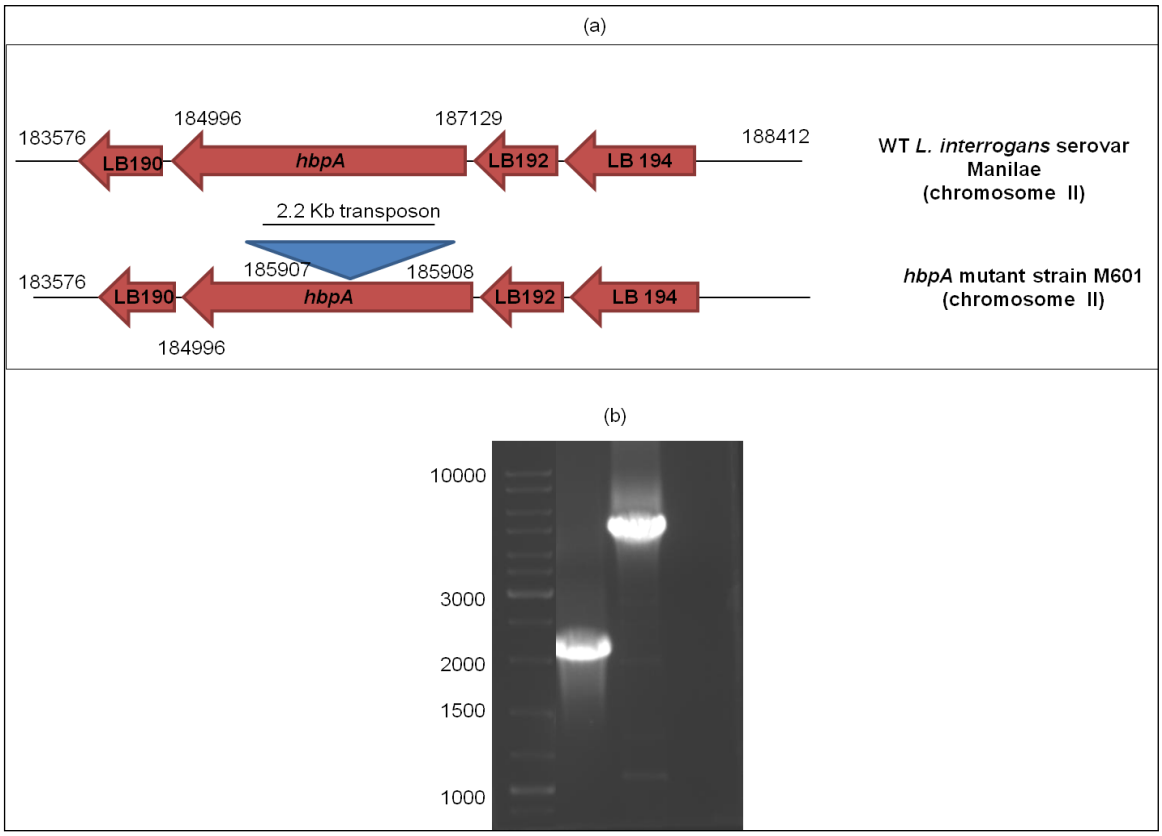


Fig. 3.17 Schematic representation of the genome of the *hbpA* mutant strain M601 of *L. interrogans* serovar Manilae. Panel (a) shows the schematic representation of the chromosome II of the *hbpA* mutant strain M601 bearing the 2.2 kb transposon inserted into the *hbpA* gene; the corresponding region in the WT genome is shown for comparison. Panel (b) shows the larger 4349 PCR product (lane 2) obtained by PCR amplification of the genome of the *hbpA* mutant strain M601 with the *hbpA* - specific full length primers. Lane 1 shows the 2149 bp product obtained with the genomic DNA from WT organisms. Lane 3 is reagent control.

The lack of expression of HbpA in the mutant strain was demonstrated by western blot analysis of the whole cell sonicate with MAb 10E3.1C. Since HbpA is an iron-regulated protein, both the WT and the *hbpA* mutant strains were grown under high and low-iron conditions. HbpA is absent in the mutant strain (Fig. 3.18a, lanes 3 & 4), while the WT organisms expressed the 81 kDa protein under low iron conditions (Fig. 3.18a, lane 2).

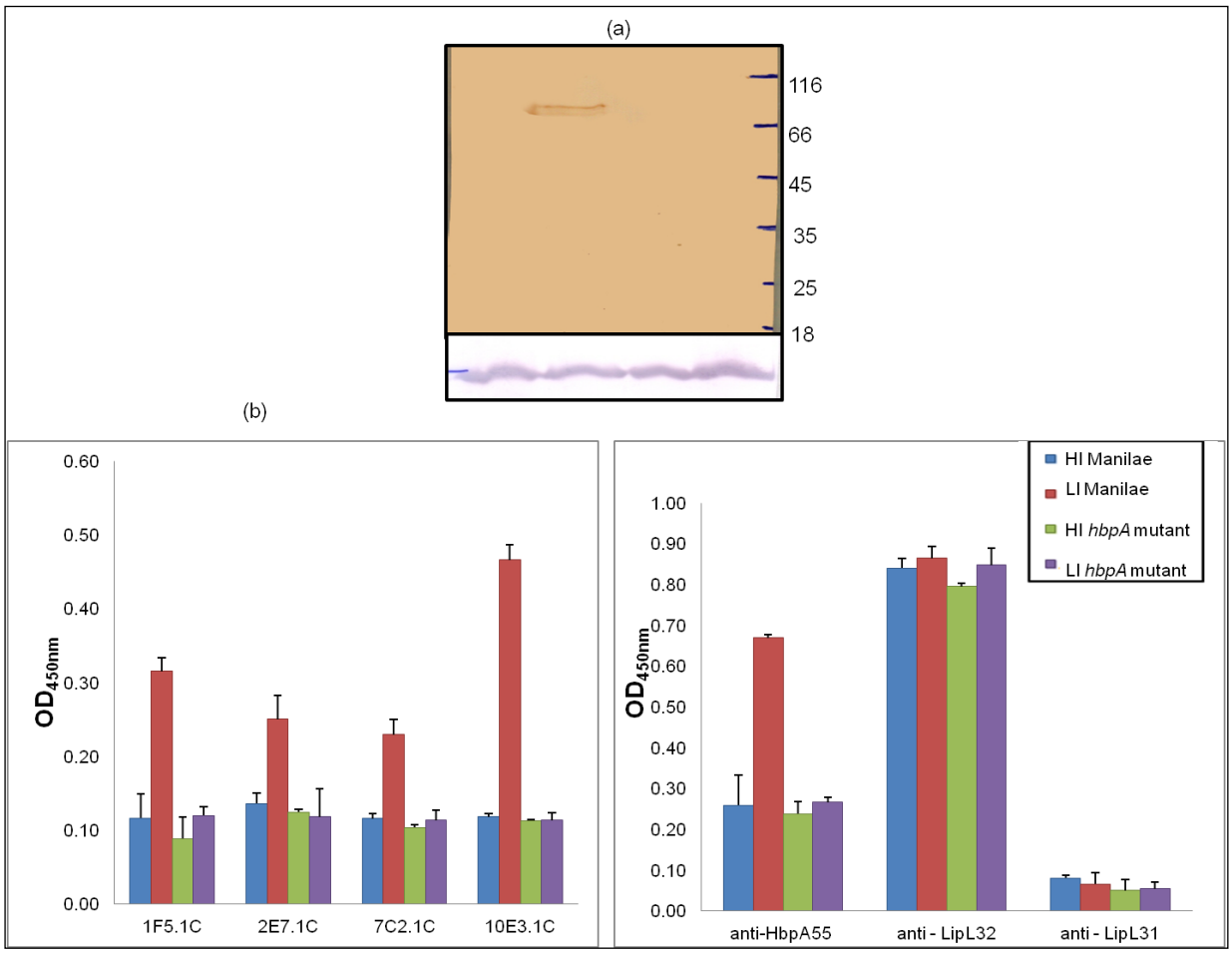


Fig 3.18 HbpA is not expressed by *hbpA* mutant strain M601 but is expressed in wild- type Manilae. In Panel (a), 60 µg of the whole cell sonicates of high (10 µg Fe ml^{-1}) and low iron grown wild-type serovar Manilae (lane 1, 2) and *hbpA* mutant strain M601 (lane 3, 4) were separated electrophoretically by SDS-PAGE and then subjected to immunoblotting with MAb 10E3.1C. The loading control is indicated by the arrow that represents the blot developed with rabbit anti-LipL41 antibody. HbpA is identified only in lane 2. M is the molecular weight marker. Panel (b) shows the ELISA-based immunoreactivity of whole cells of the mutant and WT type organisms with the four MAbs. Plates coated overnight with 10^8 cells of WT and mutant strain were incubated with 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C MAbs. Positive controls included polyclonal antibodies against $rHbpA_{55}$, LipL32 and the negative control was anti-LipL31 antibodies. Data points represent means of the experiment setup in triplicate and error bars represents standard deviations of the mean.

In addition, whole cell ELISA, performed with the MAb 10E3.1C (Fig. 3.18b) confirmed the surface expression of HbpA in low iron WT organisms and not in the mutant strain. This monoclonal antibody performed better than the other three

MAbs (1F5.1C, 2E7.1C and 7C2.1C). Antisera to the outer membrane lipoprotein LipL32 and the cytoplasmic membrane protein LipL31 were included as positive and negative controls, showing mean A_{450nm} values of 0.837 ± 0.035 and 0.063 ± 0.019 respectively. These observations not only showed the absence of HbpA in the mutant strain but also confirmed the surface expression of the protein in the WT organisms.

3.3.2. Growth kinetics of *hbpA* mutant strain M601 in iron-limited medium and hemin as sole source of iron

The growth and survival of the mutant in medium a) pre-incubated with the iron chelator EDDA and b) containing hemin as the sole source of iron were compared with the WT organism. Further, the transcriptional regulation of different iron-regulated genes were also analysed by real time PCR.

Both WT and mutant strain exhibited similar growth (Fig. 3.19) in high iron medium (10 µg Fe ml^{-1}) and grew poorly in low iron medium (in the presence of 400 µM EDDA). Interestingly, when the iron-free medium (with 200 µM EDDA) was supplemented with 1 µM hemin, WT organisms showed a dramatic increase in growth. The mutant, on the other hand, with no growth occurring for about 6 days showed a moderate increase in the number of organisms that was however much lower than the WT. The mutant strain was unable to utilise haemoglobin also as a source of iron, unlike the WT strain. EDDA at higher concentrations (400 µM EDDA) was not tolerated both by the WT and mutant, but the supplementation of hemin facilitated the growth of the WT, clearly indicating its ability to utilise the hemin.

3.3.3. Transcription regulation of genes by iron levels: identification of iron-regulated genes and *in silico* prediction of putative Fur boxes in their upstream region

Transcriptional analysis using microarrays (Lo *et al.*, 2010) of the WT *L. interrogans* serovar Manilae grown under high and low iron conditions identified 49 down-regulated genes and 43 up-regulated genes. In this study, we attempted to identify Fur boxes in the promoter regions of some of these genes, coupled to

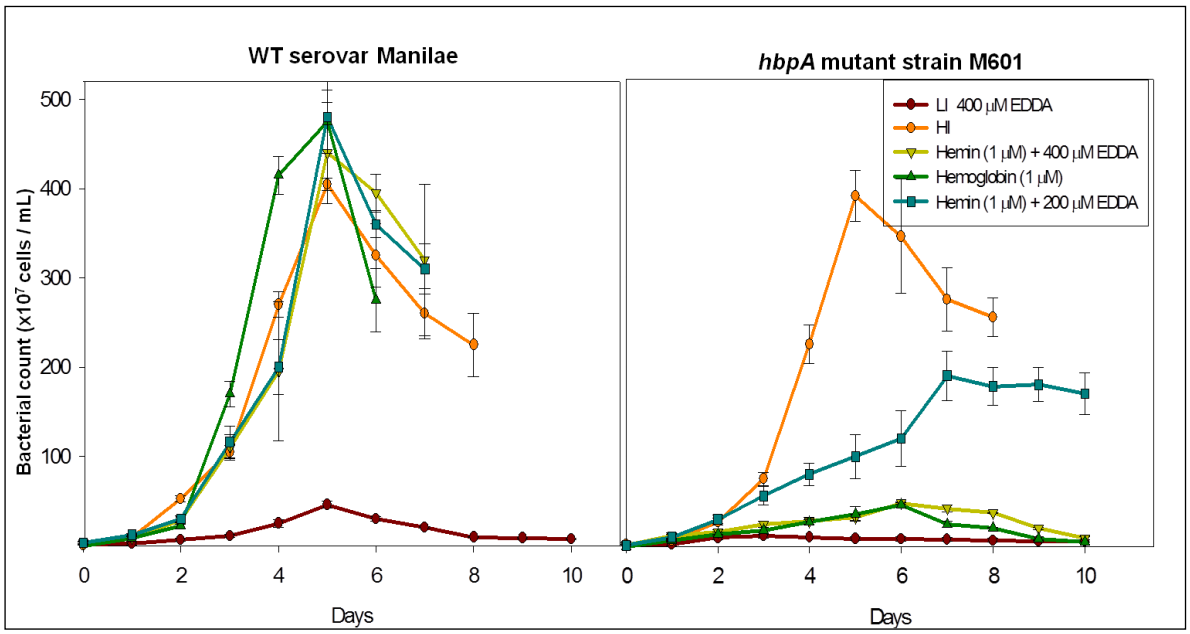


Fig. 3.19 Growth of WT *L. interrogans* serovar Manilae *and hbpA* mutant strain M601 in low iron medium and in medium with hemin / hemoglobin as source of iron. 1 x 10^7 cells of WT Manilae and the mutant strain were grown in high - (HI; 10 µg Fe mL^{-1}) and low iron (LI; 400 µM EDDA) medium. The experimental set-up included medium with 1 µM hemin / 1 µM haemoglobin added to low iron (200/ 400 µM EDDA) medium. Growth was monitored by determining the bacterial counts by dark-field microscopy. The error bars indicate standard deviation from three replicate cultures, set up under identical conditions.

which we performed qRT-PCR to confirm the findings of the microarray data, thereby understanding the influence of iron and / or HbpA on their expression.

Using PredictRegulon server, several putative Fur box sequences 300 bp upstream of the translational start site of iron-regulated genes were identified. Among them (Table 3.9), 10% constitute the reported iron-regulated genes (Lo *et al.*, 2010). Others include functionally annotated genes (32%) and genes coding for hypothetical proteins (58%) with unknown function. The Fur boxes predicted in the iron-regulated genes showed 52-78% homology to the *E. coli* consensus Fur box. Experimental validation by electrophoretic mobility gel shift assays however is necessary to confirm these genes as Fur-regulated genes.

Table 3.9 *In silico* identification of putative Fur box upstream of iron-regulated genes

Gene	Predicted function	Fold change (microarray analysis)[#]	Sequence of the Fur box	Location (relative to start site)
Fur Box in *E. coli*			GATAATGATAATCATTATC	
LA3468	TonB-dependent receptor	2.2	AATATTGAGAATCATTTTC	-24
LA2690	Bacterioferritin	-3.1	GAGAATGATAATCAAGTTC	-151
LA3469	Iron-regulated protein A	4.6	GAGAATTAGTATCAATATC	-105
LA1615	Acyl-neuraminate cytidylyl transferase	1.5	GAAAATGATTTTTGATTATA	-68
LA2242	Hypothetical protein	4.61	AATAATGAATATTATTTTA	-125
LA2062	O-acetyl homoserine sulfhydrylase	2.32	AGAAATGAAGATCAATTTC	-217
LB186 *(hemO)*	Heme oxygenase	2.49	AAAAATAAGTTTGATTCTC	-66
LB010	Glutamyl-tRNA reductase	1.40	TAAATTGAAAATTAGTCTC	-104
LB191 *(hbpA)*	HbpA	5.76	GGAAATGAATACTATTTGT	-74
LB358	Hypothetical protein	1.87	TAGAATTAAACTTAATGTC	-108

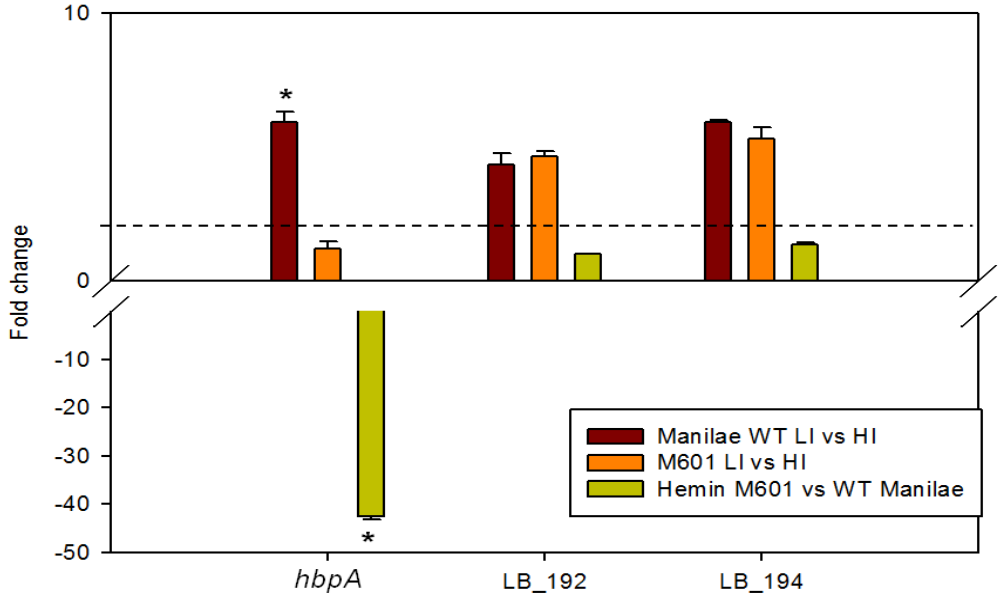
[#] (Lo *et al.*, 2010)

3.3.4. qRT-PCR of WT and *hbpA* mutant strain M601: determination of the transcript levels of selected iron-regulated genes

3.3.4.1. LB192 and LB194, encoding hypothetical proteins

LB192 and LB194 annotated as hypothetical proteins are located upstream of *hbpA* (LB191). Both the genes showed a 4-6 fold upregulation in both iron-limited WT and mutant strains (Fig. 3.20), indicating that the absence of HbpA did not

influence their expression. In the presence of hemin as iron-source, there was no significant increase in transcript levels of these genes in the mutant strain suggesting that their products are not involved in hemin uptake.

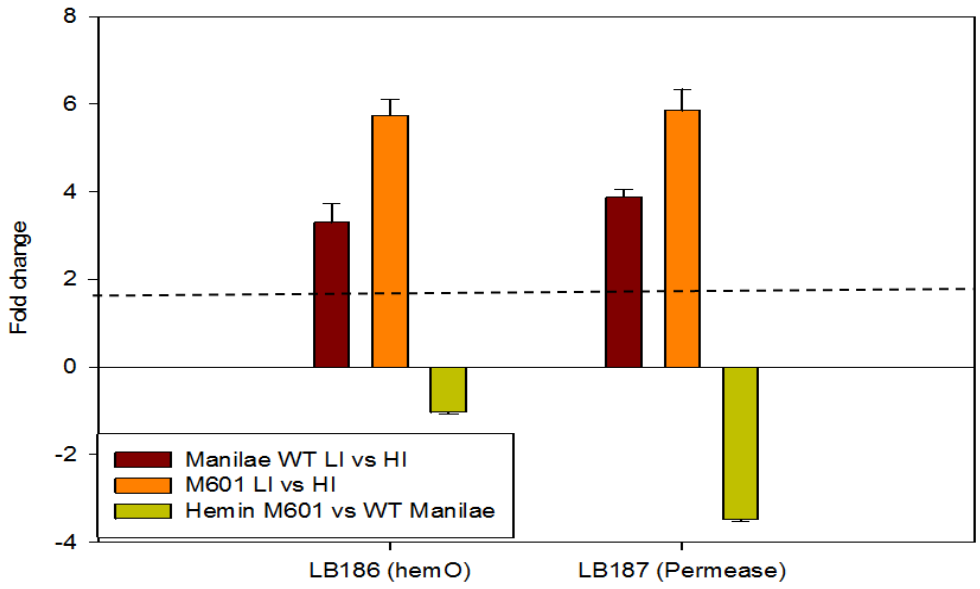


	Fold change		
	***hbpA* (LB191)**	**LB192**	**LB194**
WT LI vs HI	5.7	4.5	5.8
M601 LI vs HI	1.4	4.6	5.6
M601 vs WT (presence of hemin)	- 43	1.2	1.2

Fig 3.20 qRT-PCR: mRNA transcripts of the *hbpA* (LB191) neighboring LB192 and LB194 genes. Panel (a): RNA, isolated from high iron (HI; 10 µg Fe mL^{-1}), low iron (LI; 400 µM EDDA) and hemin (1 µM) grown organisms of WT and mutant strains was subjected to qRT-PCR using primers specific for *hbpA* (LB191), LB192, LB194 and 16S rRNA; the transcript levels were normalised with 16S rRNA. The dotted line indicates the cut-off, above which represents up-regulation and *vice versa*. Panel (b) shows the fold changes of the transcript levels of LB191, LB192 and LB194 in WT strain LI *vs* HI, mutant strain LI *vs* HI, mutant *vs* WT strains grown in the presence of hemin.

3.3.4.2. LB186 and LB187, encoding heme oxygenase and permease

LB186 and LB187, encoding heme oxygenase and permease respectively are co-transcribed together, possibly regulated by the iron regulator Fur as a Fur box is located upstream of LB186, as predicted by *in silico* analysis (see below). A ~4 fold increase in the transcripts of these two genes is seen in low iron WT strain when compared to high iron organisms; this difference is higher (~6 fold) in the mutant strain (Fig. 3.21). When hemin was used as the sole source of iron, there was a marked down-regulation of both these genes (Fig. 3.21).



	Fold change	
	LB186	**LB187**
WT LI vs HI	3.6	3.75
M601 LI vs HI	6.0	6.20
M601 vs WT (presence of hemin)	-1.06	-3.4

Fig. 3.21 Transcript levels of LB186 and LB187 in WT and mutant upon iron limitation and hemin as sole source of iron. Panel (a): RNA, isolated from high iron (HI; 10 µg Fe mL^{-1}), low iron (LI; 400 µM EDDA) and hemin (1 µM) grown organisms of WT and mutant strains was subjected to qRT-PCR using primers specific for LB186 (*hemO*), LB186 (permease) and 16S rRNA; the latter was used for normalisation of the transcript

levels. The dotted line indicates the cut-off, above which represents up-regulation and *vice versa*. Panel (b) shows the fold changes of the transcript levels of LB191, LB192 and LB194 in WT strain LI *vs* HI, mutant strain LI *vs* HI, mutant *vs* WT strains grown in the presence of hemin.

3.3.4.3. Putative iron-regulator *fur* genes

Among the four annotated Fur genes (LB183, LA1857, LA2887 and LA3094), real time PCR analysis (Fig. 3.22) showed that the transcript levels of LB183, LA1857 and LA3094 remained unaltered upon iron limitation in both WT and mutant strains (Fig. 3.22), but there was a 2.5-3 fold decrease in the transcript levels of LA2887 in both the strains grown in the presence of EDDA. When hemin was included as the sole iron source, this down-regulation was not seen and all the four fur genes showed 1-1.5 fold change in the transcripts.

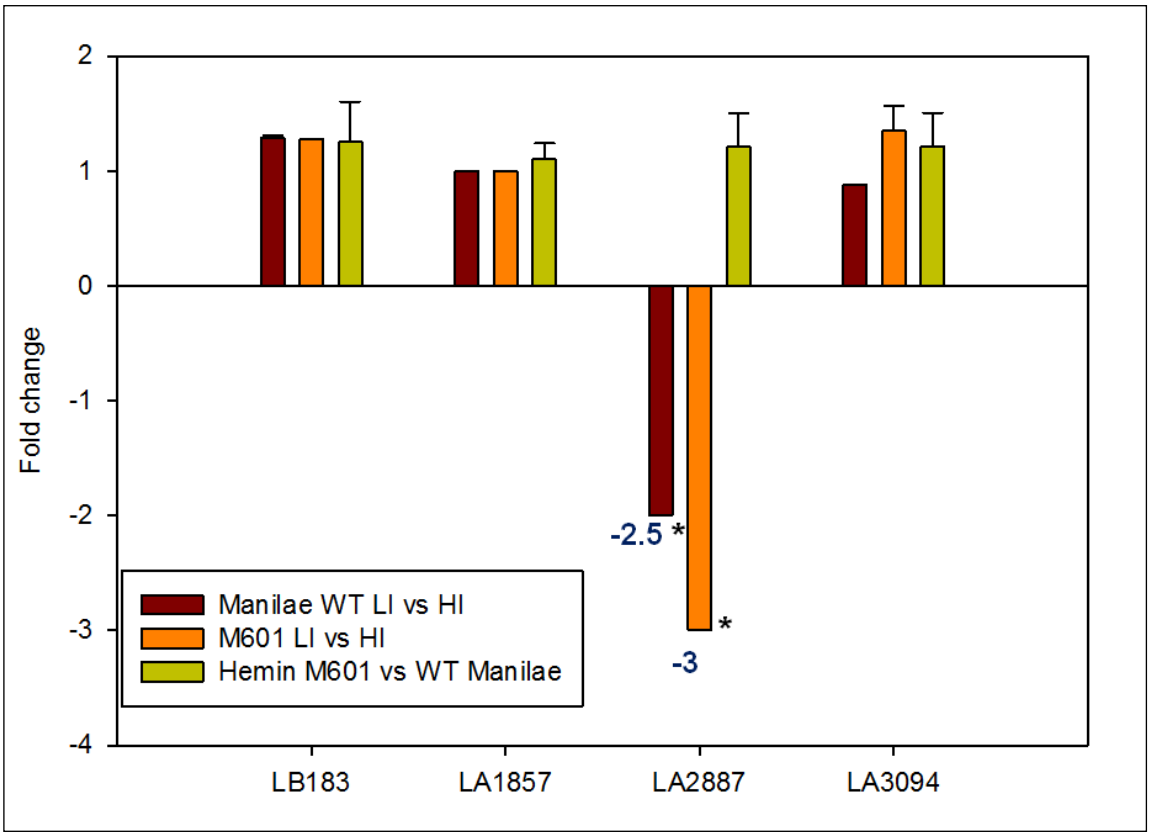


Fig. 3.22 qRT-PCR: up-regulation of the *fur* gene LA2887 and down-regulation of the iron storage bfr gene. RNA, isolated from high iron (HI; 10 µg Fe mL^{-1}), low iron (LI; 400 µM EDDA) and hemin (1 µM) grown organisms of WT and mutant strains was subjected to qRT-PCR using primers specific for LB183, LA1857, LA2887 and LA3094. The graph shows the fold changes of their levels, normalised with 16S rRNA in WT strain LI *vs* HI,

mutant strain LI *vs* HI, mutant *vs* WT strains grown in the presence of hemin. The dotted line indicates the cut-off, above which represents up-regulation and *vice versa*.

3.3.4.4. *bfr* gene encoding bacterioferritin

Bacterioferritin, encoded by *bfr* is an iron storage protein whose levels increase with an increase in cellular iron levels. Both WT and mutant strains showed high levels of *bfr* transcripts (Fig. 3.23) when grown in high iron media. In the presence of hemin, the mutant strain showed low levels of *bfr* transcripts, possibly due to its inability to acquire iron from hemin in the absence of HbpA.

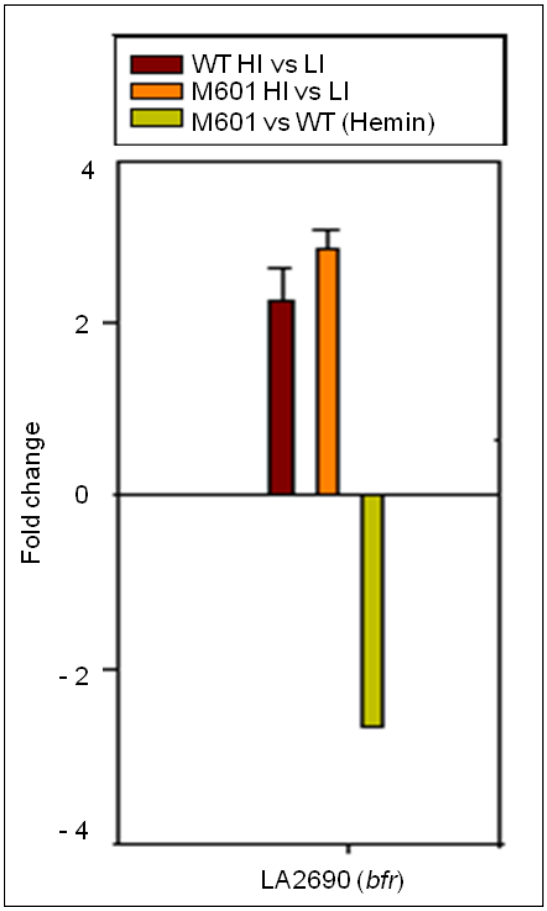


Fig 3.23 Low levels of *bfr* transcripts in the *hbpA* mutant strain supplied with hemin as the sole source of iron. RNA, isolated from high iron (HI; 10 µg Fe mL^{-1}), low iron (LI; 400 µM EDDA) and hemin (1 µM) grown organisms of WT and mutant strains was subjected to qRT-PCR using primers specific for *bfr* (LA2690). The graph shows the fold changes of their levels, normalised with 16S rRNA in WT strain LI *vs* HI, mutant strain LI *vs* HI, mutant *vs* WT strains grown in the presence of hemin.

3.3.4.5. LA3149, LA3468 and LA2242 encoding iron-regulated TonB-dependent receptor proteins

The mRNA transcripts of all the three genes were elevated 3-4 fold in both WT and mutant strain grown under low iron conditions, with a relatively higher expression induced with LA3149 (Fig. 3.24). Interestingly, the latter showed a marked 6-fold increase in the presence of hemin that was not seen with the other two transcripts (Fig. 3.24).

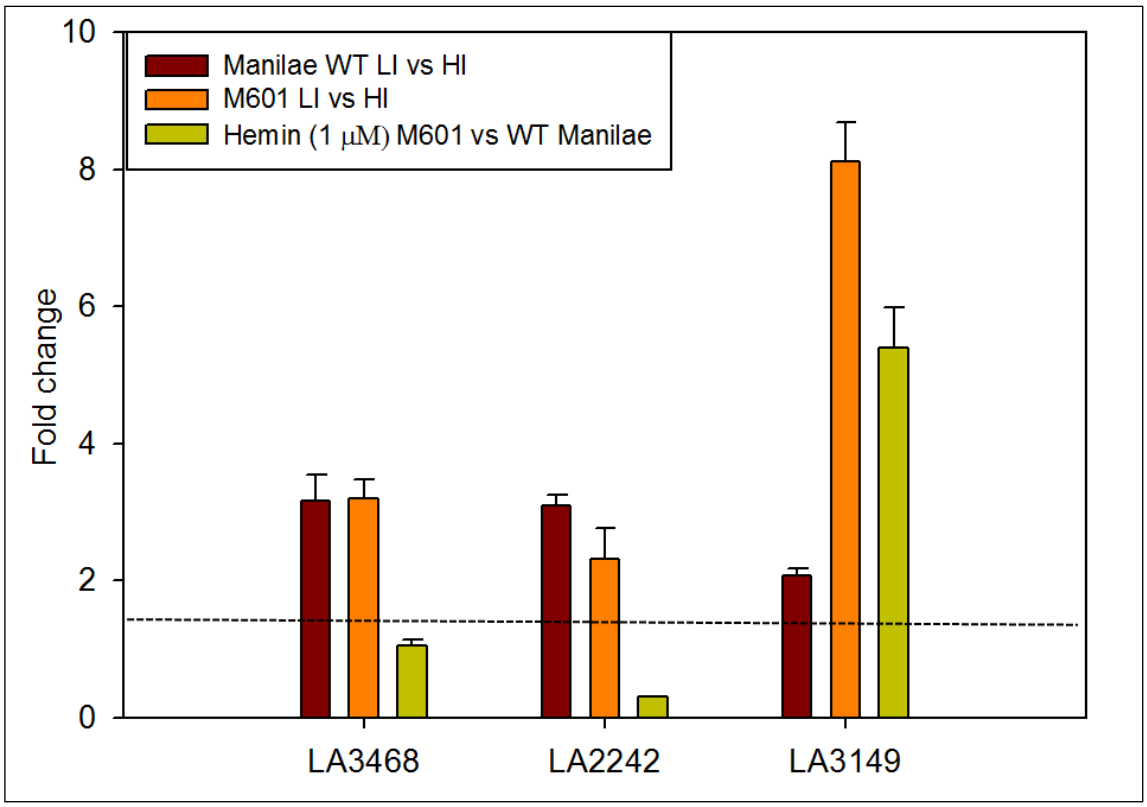


Fig. 3.24 qRT-PCR: transcript levels of LA3468, LA2242 and LA3149 encoding TonB-dependent receptor proteins. RNA, isolated from high iron (HI; 10 µg Fe mL^{-1}), low iron (LI; 400 µM EDDA) and hemin (1 µM) grown organisms of WT and mutant strains was subjected to qRT-PCR using primers specific for LA3468, LA2242 and LA3149. The graph shows the fold changes of their levels, normalised with 16S rRNA in WT strain LI *vs* HI, mutant strain LI *vs* HI, mutant *vs* WT strains grown in the presence of hemin. The dotted line indicates the cut-off, above which represents up-regulation and *vice versa*.

3.3.5. Does LA3149 encode an alternate hemin-binding protein in the *hbpA* mutant strain M601?

3.3.5.1. Time course expression of LA3149 in the presence of hemin

The transcript levels of both *hbpA* and LA3149 gene were monitored day-wise in the WT and the mutant strain grown in the presence of hemin (Fig. 2.25). There was a 3-fold increase in the transcript levels of *hbpA* gene from day 1 - 3 in WT strain, with no significant rise in the transcript levels of LA3149. In the absence of *hbpA* in the mutant, there was a marked increase in LA3149 transcript levels, reaching a 5-fold higher levels on day 4.

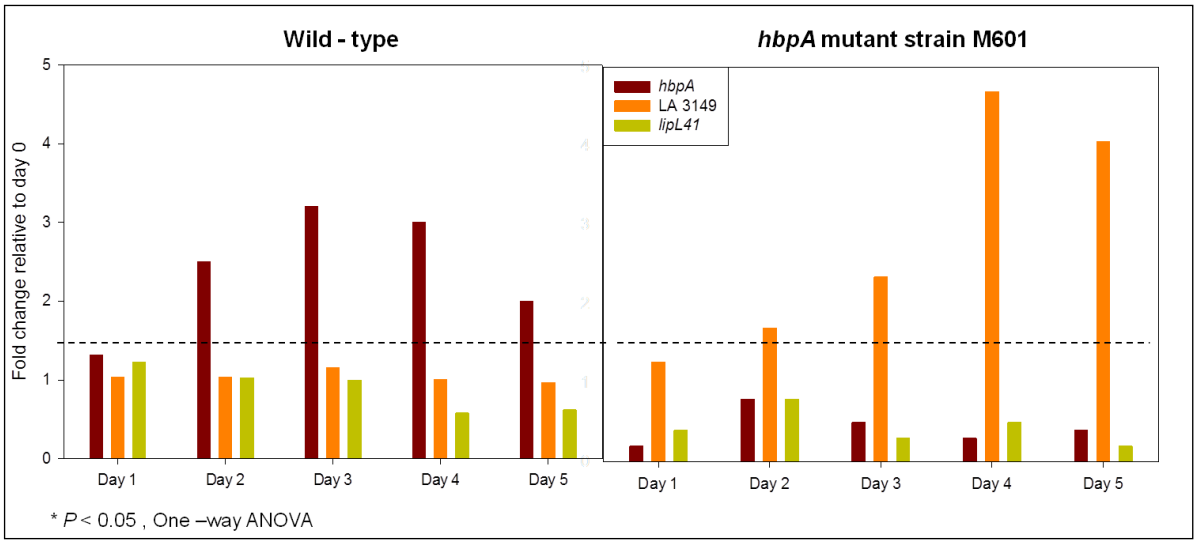


Fig. 3.25 Time course expression of *hbpA* and LA3149 in WT and hbpA mutant strain M601: qRT-PCR analysis. WT and mutant strains were grown in the presence of 1µM hemin and RNA samples from cultures harvested on days 0 – 5 were subjected to real time PCR analysis using *hbpA*- and LA3149-specific primers. The mRNA transcript levels of the genes were normalised with 16S rRNA. *lipL41* was used as the internal control. The dotted line indicates the cut-off, above which represents up-regulation and *vice versa*.

3.3.5.2. *In silico* analysis of LA3149 from serovar Lai

3.3.5.2.1. Sequence similarity and relatedness to other hemin / haemoglobin receptors

The 2334 bp LA3149 gene from *L. interrogans* serovar Lai encodes an 86 kDa protein. The protein, with a pI of 8.72 shows the presence of a signal peptide cleavage site between Ala_{25} and Leu_{26} as deduced by the SignalP program. BLAST(P) and conserved domain architecture (cdart) prediction analysis, done at NCBI (National Center for Biotechnology Information, Bethesda MD, USA) showed that, by virtue of its relatedness to the conserved domain family cd01347, it

belonged to a family of ligand-gated channel TonB-dependent proteins (e value = 9.21 e^{-54}). It is also a member of COG1629, a protein superfamily composed of outer membrane receptor proteins, specific for iron transport (e value = 1.08 e^{-42}).

When compared with other bacterial hemin / hemoglobin receptors, there was higher degree of sequence similarity in the C-terminal region (Fig. 3.26), with maximal sequence identity (21.85%) to the hemin receptor in *Porphyromonas gingivalis* HmuR. LA3149 possessed, like other hemin / hemoglobin receptors the highly conserved FRAP / NPNL and histidine residues, characteristic of the receptor domain. A FRAP-NNHL motif was identified in LA3149 between amino acid residues 536-539 and 560-563, with the histidine residue at position 550 located between the two motifs. In addition, several glutamic acid residues, conserved in hemin receptors are present in LA3149 (Fig. 3.26).

Orthologs of LA3149 are present in other pathogenic *Leptospira*, namely *L. interrogans* serovar Copenhageni (LIC10964), *L. santarosai* str. CBC613, *L. kirschneri* serovar Grippotyphosa str. RM52, *L. weilii* serovar Ranarum str. ICFT, *L. borgpetersenii* serovar Hardjo-bovis strain JB197 (LBJ 0562) and strain L550 (LBL 2518), with 99% identity in the former species compared to 84% identity in the two strains belonging to *L. borgpetersenii* (Fig. 3.27). Of clinical significance is its absence in the non-pathogenic *L. biflexa*.

The FRAP-NNHL motif and histidine residue at position 550 was conserved in all the pathogenic *Leptospira* spp., except in *L. borgpetersenii* serovar Hardjo-bovis strain JB197 and *L. weilii* serovar Ranarum wherein a KDSF and a KNDL motif was present instead of the conserved NNHL motif.

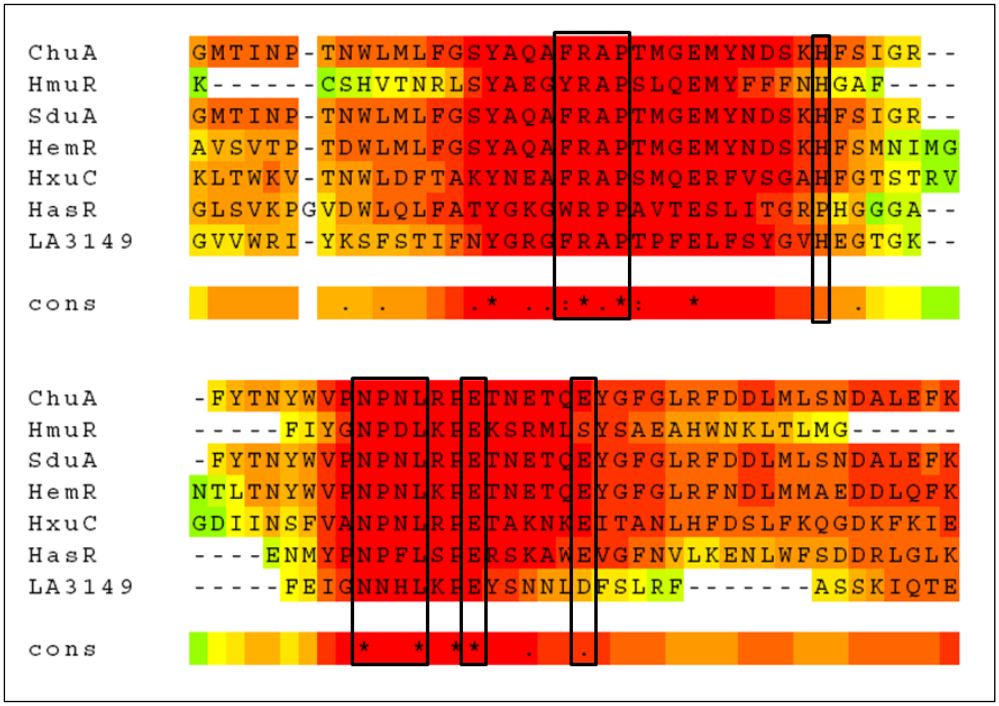


Fig. 3.26 Sequence comparison of the C-terminal end of LA3149 from serovar Lai with bacterial hemin receptors. The C-terminal region of LA3149 and hemin / hemoglobin receptors of *E. coli* (ChuA, AAG58641.1), *Porphyromonas gingivalis* (HmuR, AAQ66588.1), *Shigella dysenteriae* (Sdu, ABB63528.1), *Yersinia enterocolitica* (HemR, CAL10461.1), *Hemophilus influenzae* (HxuC, AAX87321.1), *Pseudomonas aeruginosa* (HasR, AAG06796.1) were subjected to multiple sequence alignment using T-Coffee (Ver.10.00.r1580). FRAP / NPNL and other conserved residues are boxed. Shades towards red indicate high degree of similarity with green representing residues of lower similarity. Residues that are fully conserved (*), strongly conserved (:) and weakly conserved (.) are represented in the figures.

3.3.5.2.2. Antigen-relatedness and phylogenetic analysis

Phylogenetic analysis of LA3149 with other heme receptors revealed that the protein was unique and formed a separate cluster when compared to other bacterial heme receptors (Fig. 3.28). It formed a separate cluster from HbpA (23% homology were seen between the two proteins), the latter clustering with HmuR from *Porphyromonas gingivalis*. The other bacterial hemin receptors with maximum sequence identity with LA3149 were HupO from *Vibrio fluvialis* (21.84%) and HupA from Vibrio vulnificus (20.51%).

Fig. 3.27 Sequence comparison of the C-terminal end of LA3149 from serovar Lai with orthologs from other pathogenic *Leptospira* spp. The C-terminal region of LA3149 from *L. interrogans* serovar Lai str. 56601 (Lai, NP_713329.1), *L. interrogans* serovar Manilae (Manilae, EYU62991.1), *L. santarosai* str. CBC613 (CBC613, EMK09022.1), *L. kirschneri* serovar Grippotyphosa str. RM52 (Grippo, EJO69337.1), *L. noguchii* str. 1993005606 (1993005606, EPE82196.1), *L. weilii* serovar Ranarum str. ICFT (Ranarum, EMY79307.1), *L. borgpetersenii* serovar Hardjo-bovis str. JB197 (Hardjobovis, YP_800011.1) were subjected to multiple sequence alignment using T-Coffee (Ver.10.00.r1580). FRAP / NPNL and other conserved residues are boxed. Shades towards red indicate high degree of similarity with green representing residues of lower similarity. Residues that are fully conserved (*), strongly conserved (:) and weakly conserved (.) are represented in the figures. Serovar / strain used and the accession numbers of the sequences for analysis are as given in brackets.

3.3.5.2.3. Homology modelling of LA3149

The amino acid sequence of LA3149, subjected to homology modelling using ferri-pyoverdine receptor of *Pseudomonas aeruginosa* (PDB ID: 2IAH) revealed the characteristic β-barrel structure, formed by 22 anti-parallel strands with the N-terminal globular domain, characteristic of TonB-receptors (Fig. 3.28). The

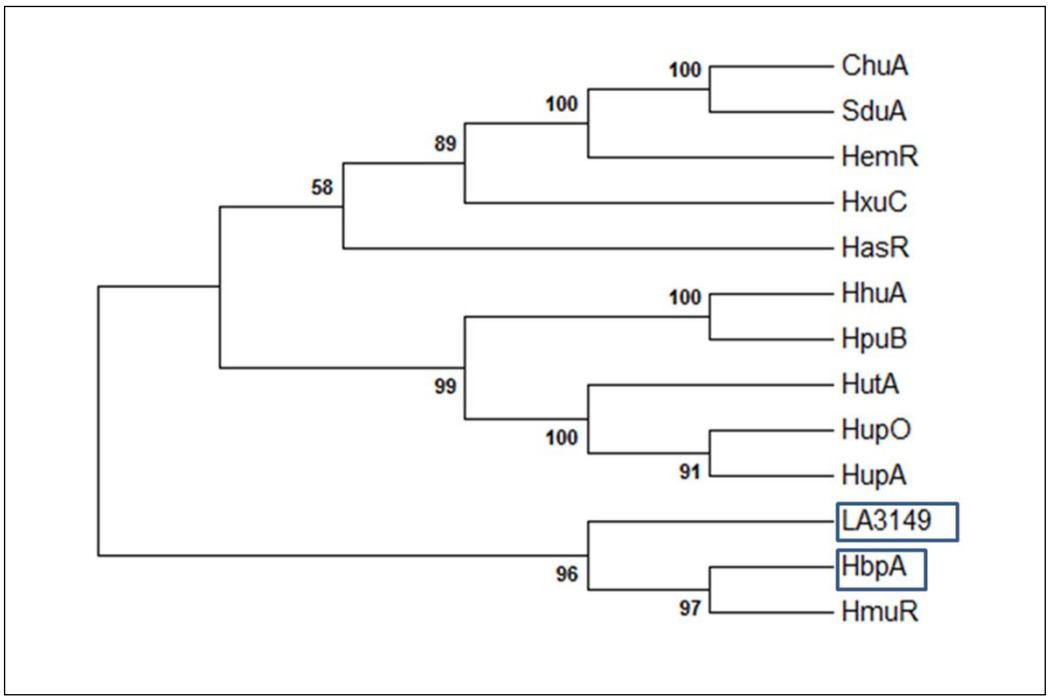


Fig. 3.28 Phylogenetic tree: antigen-relatedness of LA3149 to other bacterial hemin/ hemoglobin receptors. The MEGA software tool was used to construct the phylogenetic tree based on the amino acid sequence of LA3149 (NP_713329.1) and HbpA (LB191, AAN51750.1) from *L. interrogans* serovar Lai with other hemin / hemoglobin receptors. The latter include *E. coli* (ChuA, AAG58641.1), *Porphyromonas gingivalis* (HmuR, AAQ66588.1), *Shigella dysenteriae* (SduA , ABB63528.1), *Yersinia enterocolitica* (HemR, CAL10461.1), *Hemophilus influenzae* (HxuC, AAX87321.1), *Pseudomonas aeruginosa* (HasR, AAG06796.1), *Haemophilus influenzae* (HxuC, AAX87321.1), *Vibrio fluvialis* (HupO, AAT72471.1), *Vibrio vulnificus* (HupA, BAC15762.1), *Vibrio cholerae* (HutA, YP_002812154.1), *Haemophilus influenzae* (HhuA, AAB36696.1), *Neisseria meningitidis* (HpuB, AAC44893.2). The robustness of the tree was determined using bootstrapping with 5000 replicates.

structure was composed of 58% β strands, 6% α- helices, 2% transmembrane helices and 23% disordered structure, the latter forming the loops. Long loops were seen on the extra-cellular side, with short loops on the periplasmic side. The N-terminal region, forming the globular fits into the central pore formed by the β-barrel serves to facilitate the entry of the hemin molecule upon binding of hemin to the receptor. The structural validation of the modelled structure was validated by RAMPAGE that generated the Ramachandran plot (Fig 3.30). The latter, showing that 90.2% of peptide φ–ψ angles were within the favourable regions, with 6%

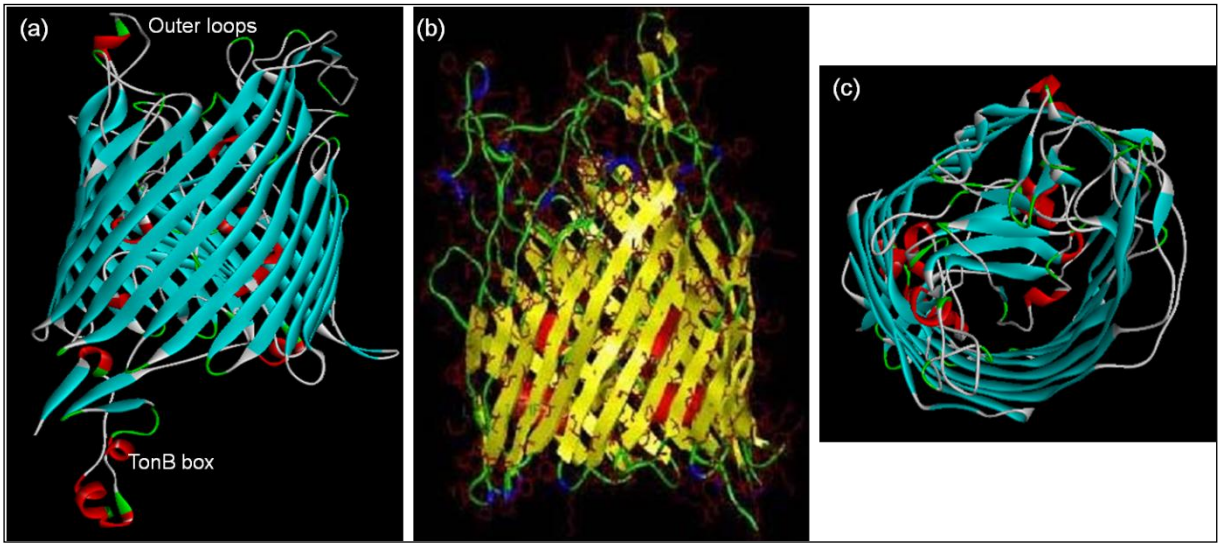


Fig 3.29 Structure prediction of LA3149 by homology modeling. Using ferri-pyoverdine receptor FpvA (PDB: 2IAH) from *Pseudomonas aeruginosa* as the template, the structure of LA3149 was modelled using Protein Homology / analogY Recognition Engine V 2.0. 696 residues. 90% of the sequence was modelled with 100% confidence by the single highest scoring template using Phyre server. Panel (a) shows the β-barrel structure with the 22 anti-parallel β strands; Panel (b) HbpA structure; Panel (c) shows the N-terminal globular plug of LA3149

falling within the additional allowed region and 3.8% in the outlier regions confirmed the stability of the modeled structure.

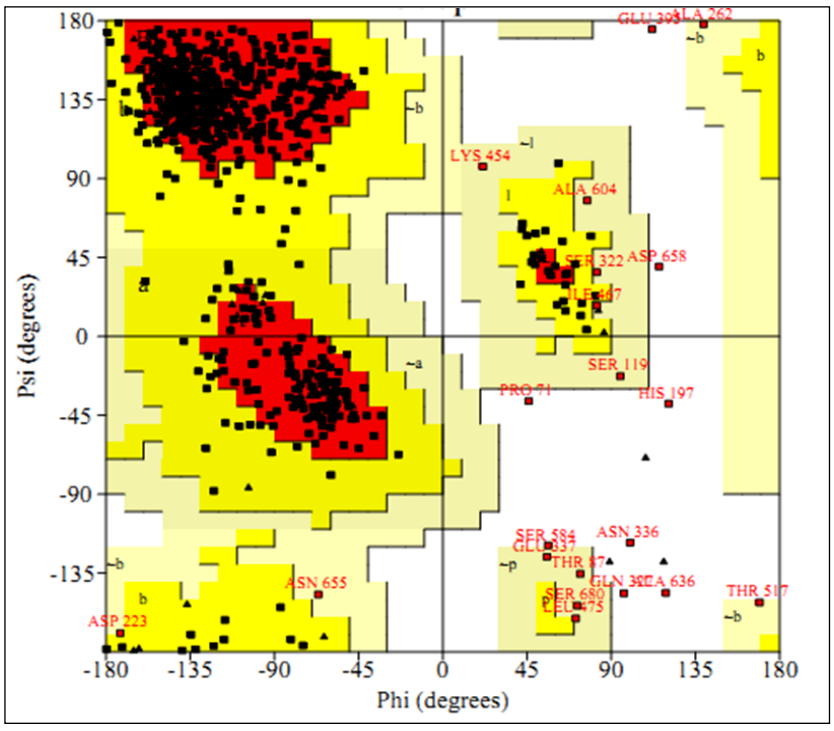


Fig. 3.30 Verification of the modeled LA3149 structure by Ramachandran plot The stability of the predicted structures was verified with the software Rampage. More than 90% of the residues were present in the favoured regions in the Ramachandran plot.

3.3.5.3. Experimental validation of LA3149 as a hemin-binding protein

A 1062 bp fragment encoding the C-terminal region of LA3149 serovar Lai, bearing the hemin-binding FRAP-NNHL motif was cloned and expressed as shown in Fig. 3.31. The recombinant protein was expressed as a 40 kDa band upon induction with IPTG, as shown in Fig. 3.31.

Whole cell sonicate of *E. coli* BL21 (DE3) expressing the 40 kDa protein was mixed with hemin-agarose beads, followed by analysis of the bound protein by electrophoretic separation on a 10% by SDS-PAGE. The 40 kDa protein (Fig. 3.31) is present only in the lane loaded with the induced sample and is absent in the un-induced *E. coli* sonicate, confirming the hemin-binding property of LA3149. Henceforth, the protein is referred to as hemin-binding protein B (HbpB).

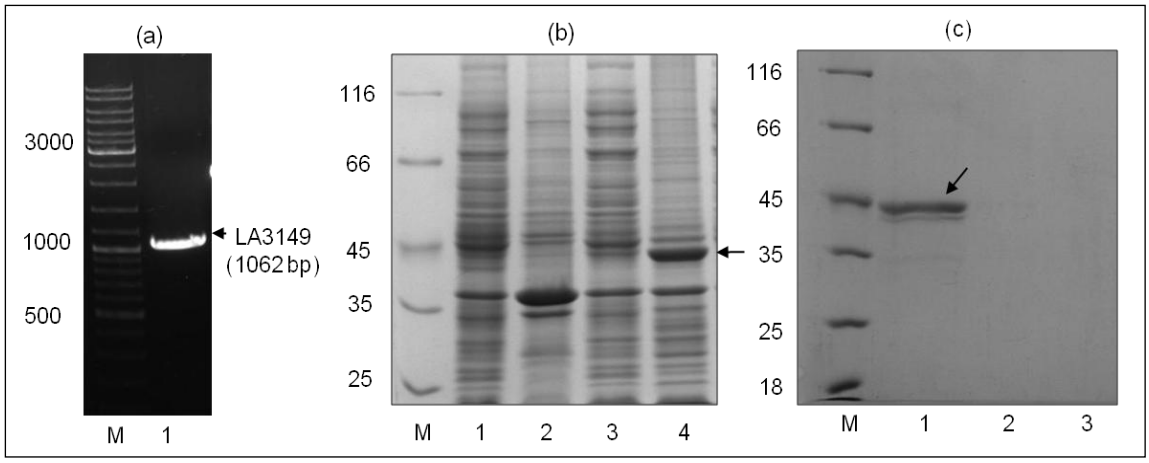


Fig 3.31 Expression and hemin-agarose binding of recombinant LA3149. Panel (a) shows the 1062 bp amplicon (lane 1) of LA3149 cloned into pET(22b) vector; M is molecular marker. Panel (b) shows the expression of recombinant LA3149 protein in *E. coli* BL21 (DE3) induced by 1 mM IPTG for 3 h; lane 1 & 2 represent whole cell sonicate of uninduced supernatant and pellet while lanes 3 & 4 represent the induced supernatant and pellet fractions respectively. Panel (c) shows the binding of LA3149 to hemin-agarose beads (lane 1; the 40 kDa band is indicated by the arrow) with lanes 2 & 3 representing hemin-agarose beads incubated with uninduced *E. coli* lysate and hemin-agarose beads alone.

CHAPTER IV

DISCUSSION

Leptospirosis is a neglected tropical disease and a public health concern worldwide. The disease has protean clinical manifestations. The classical presentation of the disease is an acute biphasic febrile illness with or without jaundice. The wide range of clinical symptoms like fever, headache, chills and myalgia overlapping with other endemic diseases like malaria, common flu, dengue fever, hantavirus infection, encephalitis and viral hepatitis makes clinical diagnosis of leptospirosis difficult (Levett, 2001). Thus, definitive laboratory diagnosis is a necessity. In addition, timely diagnosis will help to prevent the progression of the disease to the severe form, the Weil's disease that is characterised by the involvement of renal, hepatic, pulmonary, cardiovascular, neural, gastrointestinal, ocular and other systems. It took five years to decipher the leptospiral etiology of Andaman Haemorrhagic Fever (AHF) that affected more than 500 people (Sehgal *et al.*, 1995), who showed predominantly pulmonary involvement. Timely diagnosis of the disease is thus of paramount importance in initiating therapy before tissue damage occurs. Penicillin or its derivatives, namely doxycycline are highly effective in controlling the infection when initiated early in the course of illness (Faine *et al.*, 1999).

Though culture and direct demonstration of the pathogen is confirmative of the infection, the fastidious nature of the pathogen and its structural features do not allow for the easy identification of these pathogens. Serological methods are the most preferred methods of detection due to the strong humoral immunity in this disease (Bharti *et al.*, 2003). Microscopic agglutination test (MAT) is the reference gold-standard method for diagnosis of leptospirosis, which detects antibodies predominantly against the surface-exposed lipopolysacharides, which are serovar-specific, though cross-reactivity may occur against other serovars within the same serogroup (Faine *et al.*, 1999). The specificity of the lipopolysaccharide giving rise to various serovars thus necessitates the inclusion of as many as thirty or more serovars. This coupled to the technical expertise needed for the interpretation of the test and the necessity to maintain live serovars makes MAT a cumbersome test. Despite these disadvantages, MAT continues to be used as the conventional test for the diagnosis of leptospirosis. Although MAT offers high sensitivity and specificity for diagnosis of the disease, the persistence of anti-leptospiral antibodies for several years (Faine *et al.*, 1999; Levett, 2001) makes it difficult to

diagnose new infections from previous exposures. Hence, in endemic areas, a single MAT test is not sufficient and there is a need to perform the test on a second serum sample collected from the patient after 1-2 weeks; an increase in the titre in this paired sample is indicative of active infection.

Molecular tests, including PCR (Gravekamp *et al.*, 1993) and RAPD (Ramadass *et al.*, 1997) are expensive and can give rise to false positive results. Conventional PCR assay with G1/G2 primers can detect leptospiral DNA from in clinical samples (Gravekamp *et al.*, 1993). However these primers do not amplify *L. kirschneri* serovars, necessitating the use of another primer pairs B64-I / B-64-II for detection of all pathogenic serovars. These primers also however amplify non-pathogenic leptospires leading to false positive results. Therefore, the development of a diagnostic test that can detect all pathogenic serovars and can differentiate non-pathogenic from pathogenic serovars will aid in the early identification of cases of leptospirosis that remains neglected due to under-diagnosis.

Several commercial rapid serological diagnostic tests have been developed (detailed in Chapter I, Review of Literature). Among them, PanBio IgM ELISA is the most commonly used. This test, is recommended by the World Health Organization (WHO) for the serodiagnosis of leptospirosis where healthcare resources are limited (WHO, 2003). It's use in some endemic areas reflected low diagnostic accuracy (sensitivity: 36 - 60.9%; specificity: 65 - 98.0%) (Blacksell *et al.*, 2006; Effler *et al.*, 2002; Wagenaar *et al.*, 2004). The reason attributable to the poor performance may be that in countries where leptospirosis is highly endemic, a variable proportion of the apparently healthy population may be seropositive due to their repeated exposure to saprophytic *Leptospira* which causes high background titers (Desakorn *et al.*, 2012). This requires establishing an optimal tailor-made cut-off titers for tests according to different geographic regions.

Outer membrane proteins of pathogenic *Leptospira* spp., by virtue of their contact with the immediate environment have been the focus of research as potential diagnostic (and vaccine) candidates. Potential outer membrane proteins, including LipL32 (Bomfim *et al.*, 2005; Flannery *et al.*, 2001), LipL41 (Mariya *et al.*, 2006; Senthilkumar *et al.*, 2010), Lig (Croda *et al.*, 2007), LipL21 & Loa22

(Chalayon *et al.*, 2011), LruA & LruB (Verma *et al.*, 2008) have been evaluated as antigens for the sero-diagnosis of leptospirosis. Flannery *et al.,* (2001), used recombinant LipL32 antigen and reported a sensitivity of 56% in acute and 94% in convalescent phases of leptospirosis with a specificity of 90% using healthy controls (Flannery *et al.*, 2001). The LigA-based ELISA reported an 81% sensitivity in acute sera of patients with leptospirosis and a specificity of 90 - 97% (Croda *et al.*, 2007).

HbpA, first identified as a hemin-binding, TonB-dependent outer-membrane protein, (Asuthkar *et al.*, 2007; Sritharan *et al.*, 2005) has proved to be an effective diagnostic antigen. This 81 kDa protein, specific to pathogenic *Leptospira* spp. is up-regulated upon iron limitation. The iron-limiting conditions of the mammalian host, as a part of its innate immune response (Kochan, 1976) and the presence of anti-HbpA antibodies in the serum of patients with leptospirosis (Sridhar *et al.*, 2008) led us to investigate the diagnostic potential of this protein. When the genomic DNA from clinical isolates obtained from different geographical regions in the world was subjected to PCR with *hbpA*-specific primers, all serovars belonging to *L. interrogans* species were detected positive (Sridhar *et al.*, 2008). Due to the strong humoral immune response in this disease and the economic viability of an ELISA-based detection system, the latter was pursued further using HbpA as antigen. The lack of cross-reactivity of anti-HbpA antibodies with other bacterial proteins showed that HbpA was *Leptospira*-specific. The focus of the study was to evaluate the usefulness of the method for screening both human and bovine leptospirosis. This was done considering the zoonotic implications of the disease, as control measures must be enforced for the early diagnosis of the disease, thereby preventing transmission from animal hosts to humans.

Human samples included serum from patients with systemic leptospirosis and leptospiral uveitis. In the former, HbpA-ELISA was used to screen both IgM and IgG-based anti-HbpA antibodies that were compared with the conventional MAT and the PanBio ELISA. While the overall performance of HbpA-ELISA and PanBio were comparable with lower level of agreement of both the tests with MAT assay, IgG-based HbpA-ELISA proved to be superior to the detection of IgM-based antibodies against HbpA.

The HbpA-IgM and HbpA-IgG ELISA had a sensitivity of 74% and 100% respectively. A robust IgG and a poor IgM response to the HbpA antigen in the patients resulted in a higher sensitivity with HbpA-IgG when compared to HbpA-IgM ELISA. Studies using other leptospiral proteins like LipL32 (Flannery *et al.*, 2001), MPL17 and MPL21 (Oliveira *et al.*, 2008) have also documented a similar phenomenon which the authors think could possibly be due to a memory response in these individuals to a previous exposure to leptospires.

HbpA-ELISA proved to be useful in the identification of leptospiral uveitis in patients attending the Uvea Clinic in Aravind Eye Hospitals, Madurai, one of the leading eye hospitals in India. The leptospiral aetiology of uveitis is difficult to predict because of overlapping clinical symptoms with uveitis due to other causes. The findings in this study proved HbpA-ELISA to be effective in the diagnosis of leptospiral uveitis. MAT, identifying the serogroups Icterohaemorrhagiae, Australis and Autumnalis as major serovars in both Groups I (leptospiral uveitis) & II (systemic leptospirosis) patients was unable to identify 50% of the cases in Group I who presented definitive clinical symptoms of the disease, an observation also seen earlier from this geographical region (Priya et al. 2003) and reported by others (Goris *et al.*, 2012; Limmathurotsakul *et al.*, 2012). The failure of MAT is often due to the omission of the prevalent serovars in the reference panel. As epidemiological data was lacking in this region, the selection of the reference panel was based on some reported sero-prevalence studies (Koteeswaran, 2006; Kuriakose *et al.*, 1997; Mariya *et al.*, 2006; Maskey *et al.*, 2006; Natarajaseenivasan *et al.*, 2002; Sharma *et al.*, 2003; Vijayachari *et al.*, 2008).

These MAT-negative serum samples, interestingly, proved positive by HbpA-ELISA and PanBio ELISA. 92% of the patients, clinically identified as leptospiral uveitis (Group I) showed significantly high levels of anti-HbpA antibodies ($P < 0.05$ when compared to endemic controls). The performance of HbpA IgG ELISA for serodiagnosis of leptospiral uveitis with MAT and clinical diagnosis as the reference, revealed 93 and 92% sensitivity and 58 and 86% specificity, respectively. The relatively lower performance of PanBio IgM ELISA, (sensitivity and specificity of 90% and 54% with MAT as reference) was similar to other studies performed in endemic regions (Blacksell *et al.*, 2006),(Desakorn *et al.*, 2012) and (Effler *et al.*, 2002). Summarising the performance of the tests in the

diagnosis of both systemic leptospirosis and leptospiral uveitis, HbpA-ELISA was superior to PanBio IgM ELISA and both the ELISA tests were better than the conventional MAT.

HbpA-ELISA also proved successful for the detection of anti-HbpA antibodies in bovine serum samples. While all MAT positive samples were also positive by HbpA-ELISA, several MAT-negative samples tested positive by the former, for reasons mentioned earlier about the inclusion of lower number of serovars. Hence, HbpA as an antigen was compared with LipL41 and LipL32, two leptospiral antigens that showed considerable potential in the sero-diagnosis of leptospirosis. The HbpA-ELISA showed a κ value of 0.920 with LipL41-ELISA and 0.887 with LipL32-ELISA indicating good agreement of HbpA with standard leptospiral antigens. The 3 antigen-based ELISA's, showed 100% sensitivity but lower specificity (~ 63%) due to poor sensitivity of MAT.

The specificity of HbpA for pathogenic *Leptospira* and its expression *in vivo* under the physiological conditions prevailing in the mammalian host, namely at 37°C and the iron-limiting conditions make it an ideal diagnostic antigen. The simple format of ELISA would be suitable for use in routine laboratories and would be cost-effective when compared with MAT and PCR. ELISA is a better alternative to MAT as it does not require live pathogenic cultures, can achieve high sensitivity and specificity and can be semi-automated. The results can be interpreted objectively and the analysis can be done in small laboratories without the need for any specialized equipment. The assay is rapid and the antigen-coated plates can be stored and used at any time of the year, unlike MAT that requires live organisms, that are pathogenic and requires specialised growth medium that is expensive for routine laboratories. While MAT may be useful in epidemiological studies for identifying the predominant serovar, ELISA-based tests are practical for routine laboratories for quick diagnosis of leptospirosis without the need to identify the infecting serovar. The specificity and sensitivity of HbpA-ELISA, therefore offers promise as a screening test of leptospirosis.

While it is preferred to develop an antibody-based system for the screening of leptospirosis, detection of antigen has the advantage of identification of patients in the early stages of infection, before the development of anti-leptospiral

antibodies. In this endeavour, monoclonal antibodies against HbpA were generated. Using limiting cloning dilution, four clones, namely 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C were selected that secreted antibodies, specific for HbpA and which not cross react with other bacterial antigens, including the commonly encountered *E. coli*. Since His-tagged protein was used as the immunogen, we confirmed that the four MAbs that were selected were not against the His-tag by including rLipL32 / recombinant enterotoxin protein from *Clostridium spp.* with which none of the MAbs reacted. All the MAbs belonged to IgG2b subclass with MAbs 1F5.1C and 2E7.1C reacting with the recombinant proteins $HbpA_{20}$, $HbpA_{25}$ and $HbpA_{34,}$ encoding amino-acid residue 110-271, 266-454 and 421-711 respectively, while MAbs 7C2.1C and 10E3.1C reacted only with $HbpA_{34.}$

MAb 10E3.1C, used for detection of HbpA in spiked serum samples showed the highest sensitivity when compared to other MAbs as it could detect as low as 0.48 ng of rHbpA protein. Serum samples had to be spiked with HbpA as very little HbpA could be detected directly from them. This is only a preliminary study and further studies need to be done with serum collected from patients on different days after the development of symptoms of the disease. There are other reports on the use of MAbs for antigen detection. They include MAbs raised against whole-cell *Leptospira* (Ainsworth *et al.*, 1985) and LipL32 (Coutinho *et al.*, 2007; Fernandes *et al.*, 2007) and a 38 kDa leptospiral protein conserved in all pathogenic *spp*. (Saengjaruk *et al.*, 2002). While the MAbs raised against whole-cell *Leptospira* and LipL32 were not validated using clinical samples (Ainsworth *et al.*, 1985; Coutinho *et al.*, 2007; Fernandes *et al.*, 2007) the MAb LD5 against the 38 kDa protein demonstrated high sensitivity (100%; with patients on day 4 of hospitalisation) for detection of leptospiral antigen in urine of *Leptospira*-suspected patients by dot-ELISA (Saengjaruk *et al.*, 2002).

In conclusion on the applicability of HbpA as a diagnostic candidate, both antibody-based and antigen-based detection can be developed as an ELISA-based system. Further, it can be extended to the development of an easy-to-use lateral flow or dipstick assays which is economical and does not require sophisticated equipment and technical expertise.

HbpA plays an important role in the direct acquisition of iron in pathogenic *Leptospira* spp. In the event of not identifying an alternate iron acquisition system, it can be presumed to be essential for the organism. However, the generation of a mutant, defective in expressing a functional HbpA adapted itself slowly to growth in hemin-containing medium. The *hbpA* mutant strain M601 was generated by random transposon mutagenesis in *L. interrogans* serovar Manilae strain L495 (Murray *et al.*, 2009a). The *hbpA* mutant strain M601 was identified by direct sequencing of genomic DNA to locate the transposon insertions. In *hbpA* mutant strain M601, the kanamycin-resistant Himar1 transposon (2.2 kb) was inserted into *hbpA* (LB191) at position 185907 in the chromosome II (nucleotide position based on the genome of *L. interrogans* serovar Lai strain 56601), causing insertional inactivation of *hbpA*. We verified this by PCR that yielded the anticipated product of 4.3 kb in the mutant *vs* the 2.1 kb product in the WT organism. The absence of HbpA was further confirmed by immunoblotting with the MAb 10E3.1C.

The mutant strain showed similar growth pattern as the WT strain in high iron (10 µg Fe mL^{-1}) media but grew poorly in the presence of EDDA and with hemin as the sole source of iron. In the presence of the latter, however, the growth of the mutant was restored after a prolonged lag phase of about 6 days, though not attaining the cell yield as the WT strain. It clearly indicated that the loss of HbpA certainly influenced its growth in axenic cultures and it remains to be seen as to how it will survive *in vivo*. The adaptation of the pathogen, despite the decreased cell yield, indicates the presence of an alternate hemin-uptake system, as only hemin was supplied as a source of iron. Several bacterial pathogens like *Treponema denticola* (Xu & Kolodrubetz, 2002), *Vibrio vulnificus* (Datta & Crosa, 2011), *Neisseria meningitidis* (Simpson *et al.*, 2000; Stojiljkovic *et al.*, 1996) and *Pseudomonas aeruginosa* (Ochsner *et al.*, 2000) express multiple heme utilization systems. In uropathogenic *E. coli*, TonB-dependent Hma and ChuA function as heme receptors (Hagan & Mobley, 2009). Both the receptors have high affinity for hemin (K_d = 8 µM). However, Hma functions independently of ChuA to mediate heme uptake and a strain lacking both of these receptors is deficient for kidney colonization in a mouse model of urinary tract infection (Hagan & Mobley, 2009). Studies with *V. vulnificus* (Datta & Crosa, 2011) have shown two hemin uptake proteins HupA and HvtA present on the outer-membrane. However, HupA is the

major hemin uptake protein, since deletion of *hvtA* did not impair the ability of *V. vulnificus* to utilize hemin to a great extent as compared to the *hupA* mutant strain (Datta & Crosa, 2011). It is thus likely that the growth observed in *hbpA* mutant strain M601 in a culture medium containing hemin as a sole iron source is promoted by the expression of a second heme utilization system.

In our efforts to identify the second hemin-uptake system, we subjected both WT and mutant strain of *L. interrogans* serovar Manilae to growth under high, low and hemin-containing media. We established the iron status of these organisms by studying the transcript levels of reported iron-regulated genes (Lo *et al*., 2010; as studied by microarray analysis) by real time PCR. The latter clearly showed the absence of *hbpA* in the mutant, with a 5.7 fold increase in the WT strain subjected to growth under low iron conditions. In accordance with the iron-regulated expression of LB192, LB194 (both encoding hypothetical proteins), LA2690 (*bfr)*, LB186 (heme oxygenase) and LB187 (permease) as demonstrated by microarray analysis of WT organisms grown under high and low iron conditions, we confirmed their expression to be influenced by the iron levels; they were found to be unaffected by the absence of *hbpA* in the mutant strain. The up-regulation of LA2690 (*bfr*) in the WT in iron-contaning media is not surprising as Bfr is an iron storage protein. However the down-regulation of the protein in the mutant indicated clearly that the organism was unable to utilise the iron from the hemin due to the absence of HbpA. The expression of three iron-regulated TonB-dependant proteins, encoded by LA 3149 (annotated as a hemin receptor), LA3468 (FecA homolog) and LA2242 (of unknown function), when analysed showed the up-regulation of LA3149 in the mutant strain grown in the presence of hemin as the sole source of iron. This clearly indicated that the pathogen, in its efforts to acquire iron triggers the second hemin acquisition machinery, not utilised in the presence of HbpA as seen from the low levels of its transcripts in the WT strain.

Transcript levels of LA3149 were 6-fold higher in the mutant compared to the WT strain in the presence of hemin. Time course study confirmed this increase. The protein is associated with hemin-binding was confirmed by demonstrating the binding of the recombinant protein with hemin-agarose beads. Henceforth, LA3149 is referred to hemin-binding protein B, HbpB. The protein

showed the characteristic features of a TonB-dependant outer membrane receptor as seen by homology modeling of the protein using Fvp (PDB ID: 2IAH) of *Pseudomonas aeruginosa* as the template. HbpB exhibited structural similarity to HbpA (Sritharan *et al.*, 2005) and *E. coli* TonB- dependent receptors such as FepA (Buchanan *et al.*, 1999), FhuA (Ferguson *et al.*, 1998) FecA (Yue *et al.*, 2003). All these proteins showed a similar β- barrel like structure with variable extra-cellular loop regions. The β-barrel structure is relatively conserved among these proteins indicating their similar role for ligand transport across the outer membrane. The extra-cellular loops however show sequence variability among these proteins, indicating the specific function for ligand binding (Noinaj *et al.*, 2010). The presence of FRAP-NNHL motif and His_{550} amino-acid residue in HbpB, characteristic of hemin-binding proteins strengthened its-hemin binding property. Taken together, the *in silico* evidence including amino-acid similarity, domain conservation and structural similarity with other hemin/ haemoglobin binding proteins and studies with recombinant protein establishes that HbpB binds with hemin and may function as a receptor to utilise heme from serum hemoproteins.

Information of the iron-regulated expression of genes in *Leptospira* is limiting. Little is known about the Fur proteins and their binding to putative Fur boxes, normally located upstream of iron-regulated proteins. Microarray studies (Lo *et al.*, 2010) in high and low iron *L. interrogans* serovar Manilae showed no alteration in the *fur* genes (LB183, LA1857, LA2887 and LA3094). Here, real time PCR analysis showed similar results with LB183, LA1857 and LA3094 with a significant decrease in the transcript levels of LA2887 in both the strains grown in the presence of EDDA. Studies with *L. biflexa* showed a 10-fold decrease in the transcripts of LEPBIa2152 (LB183 ortholog), LEPBIa2330 (LA3094 ortholog), and LEPBIa2849 with LEPBIa2461 (LA1857 ortholog) unaffected by iron levels (Louvel *et al.*, 2006). Interestingly, the iron-regulated Fur identified in our studies has no ortholog gene in *L. biflexa*. The reason for this discrepancy in all the three studies is unknown but could possibly be due to the different growth conditions and period of growth. In addition, we predicted Fur boxes for the first time using the PredictRegulon software (Yellaboina *et al.*, 2004) and identified several Fur-regulated genes in the genome of *L. interrogans*. While some of them are known

to be iron-regulated proteins, including *hbpA*, *bfr* and *hemO*, others include proteins that need to be analysed further for their role in iron acquisition

In conclusion, this study helped to demonstrate the diagnostic potential of HbpA and the identification of a second hemin-binding protein HbpB.

CHAPTER V

CONCLUSIONS

Conclusions

HbpA is an 81 kDa TonB-dependant outer membrane protein that plays an important role in the direct acquisition of iron from the mammalian host by virtue of its hemin-binding property. In this study, HbpA was evaluated for its diagnostic potential by screening for anti-HbpA antibodies in serum from leptospirosis-infected humans and cattle, as well as screening for HbpA antigen by developing MAbs against HbpA. In addition and of interest, is the identification of a second hemin-binding protein HbpB in *L. interrogans* serovar Manilae strain M601, in which *hbpA* was disrupted by transposon insertional mutagenesis. The following are the salient observations from the study

I. Evaluation of the diagnostic potential of the hemin-binding protein HbpA

A. Screening of serum samples for anti-HbpA antibodies by HbpA-ELISA

1) HbpA-ELISA was standardized using $HbpA_{55}$ as antigen. This 55 kDa protein contains the C-terminal end of the protein containing the residues for hemin-binding.
2) Using 250 ng $HbpA_{55}$ as antigen and serum (diluted 1:200) from patients suspected of systemic leptospirosis, HbpA-ELISA was performed to detect both IgG and IgM-specific anti-HbpA antibodies. The findings compared with the conventional MAT and the commercial PanBio IgM ELISA showed the superior performance of HbpA-ELISA over both MAT and PanBio ELISA. In HbpA-ELISA, the IgG class screening showed higher sensitivity (100%) and specificity (100%) using MAT as the reference test. Screening for IgM class of anti-HbpA antibodies gave a sensitivity and specificity of 74% and 100% respectively. PanBio IgM ELISA with a sensitivity and specificity of 88% and 73% respectively performed better than MAT.
3) HbpA-ELISA was evaluated in a study conducted from samples from study subjects from Aravind Eye Hospital, Madurai to evaluate its usefulness in identification of leptospiral uveitis. The study included five study groups: leptospiral uveitis (Group I), systemic leptospirosis (Group II), non-leptospiral uveitis (Group III), cataract cases (Group IV) and healthy endemic controls (Group V). Salient observation was that HbpA-ELISA and PanBio ELISA detected a majority of the clinically proven leptospiral uvetis cases, while MAT

identified only 50% of them as positive, with HbpA-ELISA performing better (92% positivity) than PanBio IgM ELISA (82%). The mean titre of anti-HbpA antibodies was significantly higher in the patients as compared to controls ($P < 0.05$). While Group II served as the positive controls with good correlation of the two ELISA tests with MAT, all the other three groups showed negligible titres, establishing the usefulness of HbpA-ELISA in the screening of leptospiral uveitis.

4) HbpA-ELISA proved effective in the screening of bovine leptospirosis. A panel of bovine serum samples, grouped into MAT positive and negative were evaluated by ELISA using three leptospiral antigens, namely HbpA, LipL41 and LipL32. The test showed a κ value of 0.920 for HbpA-ELISA vs LipL41-ELISA and 0.887 for HpbA-ELISA vs LipL32-ELISA indicating good agreement of HbpA with the two leptospiral antigens. All the three antigen-based ELISA tests showed 100% sensitivity with MAT; the specificity, however was low (~ 63%), for reasons of not including a larger panel of serovars.

B. Generation of monoclonal antibodies against HbpA of *L. interrogans* serovar Lai and evaluation of their potential in detection of HbpA in serum samples

1) Four monoclonal antibodies 1F5.1C, 2E7.1C, 7C2.1C and 10E3.1C secreting HbpA-specific antibodies were generated.
2) All the four MAbs reacted with $HbpA_{55}$, while MAbs 7C2.1C and 10E3.1C showed specific reactivity with $HbpA_{34}$, containing the C – terminal (aa 421-711) amino acids of HbpA
3) HbpA detection was done with serum spiked with varying concentration of r $HbpA_{55}$. MAb 10E3.1C detected as low as 0.48 ng of r $HbpA_{55}$.
4) MAb 10E3.1C detected antigen in serum samples from patients with leptospirosis; due to the low levels detected, the antigen levels were confirmed upon spiking the serum samples with 1 ng of r$HbpA_{55.}$

II. Identification of a second hemin-binding protein HbpB (LA3149) in *L. interrogans* serovar Manilae

1) A second hemin-binding protein HbpB was identified in a *hbpA* mutant strain M601.

2) HbpB is an 86 kDa protein encoded by LA3149.
3) A truncated HbpB, bearing the C-terminal amino acids (424 to 778) containing the residues needed for hemin-binding was expressed as a recombinant protein. The rHbpB (40kDa) was demonstrated to bind hemin.
4) Homology modeling showed HbpB to possess the characteristic β-barrel structure characteristic of several hemin-binding / ferric-siderophore receptors. The other features indicative of the hemin-binding property of HbpB is the presence of the FRAP-NPHL. The annotation of the protein as a TonB-dependant protein was evident by the presence of the TonB box in the N-terminus.

In conclusion, the diagnostic potential of HbpA was established in this study and a second hemin-binding protein HbpB was identified in the pathogenic serovar *L. interrogans* serovar Manilae, both of which are important contributions in the field of leptospirosis.

CHAPTER VI

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CHAPTER VII

PUBLICATIONS

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An ELISA for leptospiral hemin-binding protein antibody in bovine serum

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ABSTRACT

Sivakolundu S, Nagendrakumar SB, Suneel NA, Chandran D, Sritharan M., ELISA for leptospiral hemin-binding protein antibody in bovine serum, Online J Vet Res., 15(6): 476-484, 2011. Leptospiral hemin-binding protein antibody (HbpA) is an iron-regulated hemin-binding protein in several pathogenic serovars but absent in saprophytic leptospires. The diagnostic potential of HbpA for cattle was explored since it induces anti-HbpA antibodies in human serum. An ELISA-based assay for the detection of anti-HbpA antibodies in serum was used to screen 176 bovine serum samples. The test was validated against the microscopic agglutination test (MAT) and standard leptospiral antigens LipL32 and LipL41. The test showed a κ value of 0.920 for HbpA-ELISA vs LipL41-ELISA and 0.887 for HpbA-ELISA vs LipL32-ELISA indicating good agreement of HbpA with standard leptospiral antigens. The 3 antigen-based ELISA's, showed 100% sensitivity but lower specificity (~ 63%) compared with MAT. The lower specificity may have been due to a limited number of serovars for MAT screening. Results suggest that the described ELISA may have an advantage for detection of anti-leptospiral antibodies over MAT, which requires a large number of live leptospiral serovars.

Keywords: Leptospirosis, bovine, HbpA, LipL41, LipL32, ELISA

INTRODUCTION

Leptospirosis is a zoonotic disease caused by the pathogenic serovars of the spirochaetal bacteria belonging to the genus *Leptospira.* It has worldwide distribution and affects humans, livestock and wild animals (Levett, 2001). In cattle, the clinical manifestations of the disease range from acute symptoms to chronic disease that could result in abortion, stillbirth, infertility, decreased milk production and death (Ellis, 1984). Thus, the disease causes considerable economic loss in the dairy industry. Bovine leptospirosis is of major concern, not only due to its transmission to other animals in the herd but due to the zoonotic implications to humans working in close contact with the infected animals, especially the livestock farmers (Adler et al., 2010).

Control measures for leptospirosis include timely diagnosis and treatment. Diagnosis of the disease is conventionally done by the 'microscopic agglutination test' (MAT). The test, based on the agglutination of live leptospires by anti-leptospiral antibodies in the serum of suspected animal / human host is considered as the gold standard for the diagnosis of the disease. Due to several disadvantages of this method, there is a need to develop a simple, easy-to-perform immunoassay for the detection of antibodies against pathogen-specific leptospiral antigens expressed *in vivo*.

HbpA, first demonstrated in *L. interrogans* serovar Lai (Sritharan et al., 2005; Asuthkar et al., 2007) is an 81 kDa iron-regulated hemin-binding protein expressed under conditions of iron limitation. The *hbpA* gene, encoding the protein is present in several pathogenic serovars and is absent in the saprophytic leptospires, making it an ideal candidate for diagnosis. This was demonstrated by PCR amplification of the gene from the genomic DNA of several clinical isolates (Velineni et al., 2008). The presence of anti-HbpA antibodies in the serum of human patients with leptospirosis (Velineni et al., 2008), indicating the *in vivo* expression of the protein led us to develop an enzyme-linked immunosorbent assay for detecting anti-HbpA antibodies in the serum of cattle and validate it against MAT. HbpA-ELISA was also compared with ELISA using the leptospiral antigens LipL41 and LipL32, whose diagnostic potential was established by other studies (Bomfim et al. 2005; Mariya et al., 2006; Senthilkumar et al., 2009).

MATERIALS AND METHODS

Bacterial strains and growth: The leptospiral reference strains (Table 1) were obtained from the Regional Medical Research Centre and WHO Collaborating Center for Diagnosis, Reference, Research and Training in Leptospirosis (ICMR), Port Blair, Andaman and Nicobar Islands and maintained in semi-solid (0.15% Noble agar) EMJH medium supplemented with 10% enrichment (Difco, USA) at 30°C.

Serum samples: 176 bovine serum samples collected from various geographical regions of India were obtained from Indian Veterinary Research Institute, Izatnagar, India (a kind gift from Dr. S. K. Srivastava).

Microscopic Agglutination Test (MAT): This was done as per published protocols (Cole et al., 1973) using 12 different serovars as listed in Table 1. The end point was taken as the highest dilution of the serum in which 50% of the organisms were agglutinated or there was a 50% reduction in the number of organisms as compared to control. Titres ≥ 100 were considered as positive.

Table 1: *Leptospira* strains

Species	Serogroup	Serovar	Strain
L. interrogans	Autumnalis	Bankinang	BankinangI
	Canicola	Canicola	Hond Uttrecht IV
	Sejroe	Hardjo	Hardjoprajitno
	Australis	Australis	Ballico
	Hebdomadis	Hebdomadis	Hebdomadis
	Icterohaemorrhagiae	Lai	Lai
	Pomona	Pomona	Pomona
L. borgpetersenii	Tarassovi	Tarassovi	Perepelicin
	Ballum	Ballum	MUS127
	Javanica	Poi	Poi
L. meyeri	Ranarum	Ranarum	ICF
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V
L. biflexa	Semaranga	Patoc	Patoc-I
L. santarosai	Sarmin	Weaveri	CZ-390

Cloning, expression and purification of rHbpA, rLipL41 and rLipL32 for use as antigens in ELISA: Cloning and expression of rHbpA antigen was done as reported earlier (Asuthkar et al., 2007). The genes encoding LipL41 and LipL32 were cloned into the bacterial expression vector pRSET A (Invitrogen, USA), transformed into BL21(DE3) pLysS (Invitrogen, USA), induced using 1 mM isopropyl β-D thiogalactoside (IPTG) and expressed as C-terminal 6xHis extensions with molecular weights of ~41 kDa and ~32 kDa respectively. All the three recombinant proteins were purified under denaturing conditions using commercial Ni-NTA His-bind columns (Novagen) as per manufacturer's instructions.

ELISA: Using standard protocol (Flannery et al., 2001), each serum sample was tested for the presence of antibodies against HbpA, LipL41 and LipL32 respectively. Optimal concentration of the recombinant protein(s) and the serum dilutions were determined by checker board titration. Flat-bottomed polystyrene microtitre plates (Corning) were coated with rHbpA / rLipL41 / rLipL32 proteins (100 µl in 0.1 M bicarbonate buffer, pH 9.2) for 3 h at 37°C, followed

by overnight incubation at 4°C. The plates were washed three times with 10 mM PBST (phosphate-buffered saline: 0.01 M phosphate buffer pH 7.4 with 0.05% Tween-20 and 0.9% NaCl) followed by blocking with 5% (w/v) BSA in PBST for 2 h at 37°C. 100 µl of serum (1:200) was added and incubated for 2 h at 37°C. After washing, the plates were incubated for 2 h at 37°C with 100 µl of rabbit anti-bovine IgG alkaline phosphatase conjugate (1:500 dilution; Bangalore Genei, India). 100 µl of the substrate p- nitrophenyl phosphate (BioRad, USA) was added, incubated for 15 min and the absorbance of the developed color was measured at 405 nm in an ELISA reader (Model 680XR, BioRad, USA). Antigen and antibody blanks were included in the test to check for background colour. A known positive serum sample and normal bovine serum were used as positive and negative controls respectively. All the serum samples were tested in triplicate.

Statistical analysis

A receiver operating characteristic curve (ROC) (MedCalc Software, Mariakerke, Belgium) (Schoonjans et al., 1995) was used to calculate the cut-off value, sensitivity, specificity, area under curve (AUC), 95% CI (confidence intervals) and others to determine the diagnostic value of an antigen.

Kappa statistics (Altman, 1995) was used to assess the degree of agreement of HbpA-ELISA with LipL41-ELISA and LipL32-ELISA and the κ values obtained were used to grade the performance as follows: poor (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), good (0.61–0.80) and very good (0.81–1.00).

RESULTS

Based on a panel of 12 serovars, 41 of the 176 bovine serum samples (23%) were demonstrated to be positive by MAT. Among the positive samples, 46% were specific for *L. interrogans* serovar Canicola; the rest included Pomona (19.51%), Hardjo (19.51%), Lai (12.19%) and *L. borgpetersenii* serovar Tarassovi (2.4%).

Standardisation of ELISA was initially done by optimization of the antigen concentration and suitable dilution of the known positive control serum sample so that maximal difference was obtained between the positive and negative controls. All the test serum samples (diluted 1:200) were screened using 250 ng / well of rHbpA and 50 ng / well of rLipL41 and rLipL32. Table 2 lists the performance of the above three antigens in ELISA as compared to MAT.

Table 2 – Evaluation of ELISA-based leptospiral diagnosis against MAT

		MAT		Total
		Positive	Negative	
HbpA	Positive	41	50	91
	Negative	0	85	85
Total		41	135	176

		MAT		Total
		Positive	Negative	
LipL32	Positive	41	42	83
	Negative	0	93	93
Total		41	135	176

		MAT		Total
		Positive	Negative	
LipL41	Positive	41	47	88
	Negative	0	88	88
Total		41	135	176

Using the MedCalc Software, ROC curves for the three antigens (Fig. 1) and statistical data (Table 3) were generated. Samples detected positive by MAT were also positive by ELISA (Table 2) thus reflecting a sensitivity of 100% of all the three antigen-based ELISA with MAT (Table 3). The specificity, however was low (≥ 63 %, Table 3), the reason for which is discussed below. The cut-off value, representing the maximal sum of sensitivity and specificity was determined to be 27.94% for HbpA (Table 3) and all values above this cut-off was considered as positive. A similar analysis for LipL41 and LipL32 showed that all the three antigens showed a higher positivity than MAT (% positivity of 51.7, 50 and 47.15 for HbpA, LipL41 and LipL32 respectively).

Table 3: ROC curve analyses of ELISA vs MAT

Parameter	**Antigens used in ELISA**		
	HbpA	**LipL32**	**LipL41**
AUC (95% CL)[a]	0.909(0.856-0.947)	0.866(0.807-0.913)	0.841(0.779-0.892)
Standard error	0.032	0.037	0.040
Cut-off (%)	27.94	23.51	32.28
% Sensitivity (95% CL)[a]	100 (91.4-100.0)	100 (91.4-100.0)	100 (91.4-100.0)
% Specificity (95% CL)[a]	62.96 (54.2-71.1)	68.89(60.4-76.6)	65.19 (56.5-73.2)
Positive predictive value (%)	45.05	49.39	46.59
Negative predictive value (%)	100	100	100

[a]95% confidence limits

Fig.1

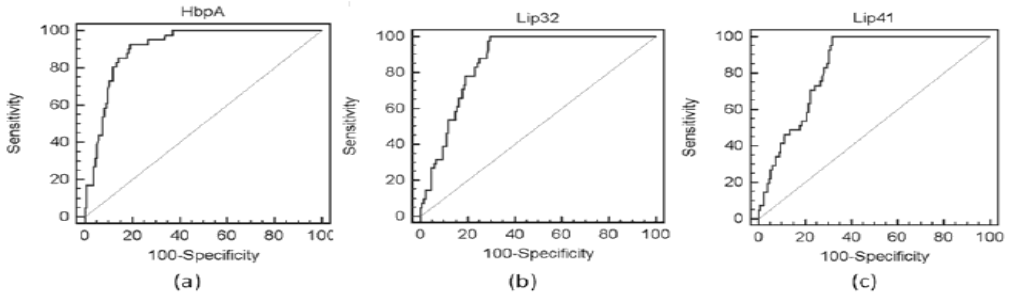

Fig. 1 - ROC curves. The ROC curves were generated from the MAT and ELISA data of HbpA (a), LipL32 (b) and LipL41 (c). The sensitivity was plotted against 100-specificity to determine the cut-off for analysis

As the ELISA-based detection of anti-leptospiral antibodies was more promising than MAT, the degree of agreement among the three antigen-based ELISA was evaluated by Kappa statistical analysis (Table 4); κ value of 0.920 for HbpA vs LipL41 and 0.887 for HbpA vs LipL32 indicated very good agreement among the three antigens.

Table 4 – Comparison of HbpA vs LipL32 and LipL41 in the sero-diagnosis of leptospirosis using κ statistics

		LipL41		LipL32	
		Positive	Negative	Positive	Negative
HbpA	Positive	86	5	81	10
	Negative	2	83	1	84

Serological tests	κ value (95% CI)
HbpA vs LipL32 ELISA	0.887 (0.819- 0.955)
HbpA vs LipL41 ELISA	0.920 (0.863- 0.978)

DISCUSSION

Outer membrane proteins in pathogenic *Leptospira* spp. have been the focus of research as potential vaccine and diagnostic candidates as they are surface-exposed and are in contact with the immediate environment of the mammalian host. Several outer membrane proteins including LipL32 (Bomfim et al. 2005), LipL41 (Mariya et al., 2006; Senthilkumar et al., 2009), LigB (Sankar et al., 2010), LipL21 & Loa22 (Chalayon et al., 2011), LruA & LruB (Verma et al., 2005) and LigA (Srimanote et al., 2008) have been evaluated as antigens in the sero-diagnosis of leptospirosis. Studies in our lab demonstrated the diagnostic potential of the leptospiral hemin-binding protein HbpA (Velineni et al., 2008). Here, an ELISA-based protocol for the detection of anti-HbpA antibodies in bovine serum samples was validated against the conventional diagnostic test MAT. In addition, the performance of HbpA was compared against the leptospiral antigens LipL41 and LipL32 in ELISA.

HbpA is a TonB-dependant outer membrane protein expressed by iron-limited *L. interrogans* serovar Lai (Asuthkar et al., 2007). This cell surface-expressed protein functions as a hemin-binding protein, thereby enabling the pathogen, lacking the ability to synthesise siderophores to acquire iron directly. HbpA would certainly play an important role in the survival of the pathogen within the mammalian host, as the latter limits iron for an invading pathogen by a process called as nutritional immunity (Kochan, 1976). It was therefore not surprising to detect anti-HbpA antibodies in the serum of patients with leptospirosis (Velineni et al., 2008). The expression of HbpA *in vivo* (Velineni et al., 2008), the absence of hbpA in the non-pathogenic Leptospira spp. (Velineni et al., 2008) and PCR-based detection of *hbpA* in clinical isolates (Asuthkar et al., 2007, Velineni et al., 2008) led us to develop an ELISA-based method for the detection of anti-HbpA antibodies in the serum of cattle (this study) and humans (manuscript under preparation).

HbpA-ELISA identified all the 41 MAT-positive samples accounting for 100% sensitivity. However, the detection of anti-HbpA antibodies in several MAT – negative samples reflected the low specificity of HbpA-ELISA vs MAT. The failure of MAT to detect anti-leptospiral antibodies could be attributed to the inclusion of only twelve leptospiral serovars in the study. The selection of this panel of serovars was based on a few sero-prevalence studies (Koteeswaran, 2006; Kuriakose et al, 1997; Mariya et al., 2006; Maskey et al., 2006; Natarajaseenivasan et al., 2002; Sankar et al., 2010; Sharma et al., 2003; Vijayachari et al., 2008) as no planned study has been conducted in India for the identification of the prevalent serovars in the country. As MAT is specific, detecting only closely-related serovars within a serogroup, it is likely that the test serum samples, with antibodies against serovars not included in the test were categorized as MAT – negative. This problem can be overcome by the inclusion of a larger panel of leptospiral serovars; the latter however is expensive and labor-intensive.

The study reflects the superior performance of ELISA over MAT in the detection of anti-leptospiral antibodies, as statistically significant results were obtained with ELISA based on LipL41 and LipL32 respectively. Further, it has been reported that active bovine leptospiral infection can occur in the absence of detectable agglutination titre in MAT (Ellis et al., 1982)

which could result in more samples being ELISA positive than MAT. It would be appropriate to correlate diagnosis of the disease, not with just MAT, but also with clinical symptoms and culture.

CONCLUSION

In conclusion, HbpA can be used alone or in combination with the other two leptospiral antigens in an ELISA for the serodiagnosis of leptospirosis. ELISA-based testing will be less expensive than MAT as it does not require the maintenance of live organisms and can be performed in any routine laboratory while a major drawback of MAT is that the analysis requires trained personnel and is prone to inter-observer and intra-observer errors.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Serological diagnosis of leptospiral uveitis by HbpA IgG ELISA

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Leptospirosis is a zoonotic disease that is highly prevalent in tropical countries; uveitis is one of the manifestations of leptospirosis. The leptospiral aetiology of uveitis is difficult to predict because of overlapping clinical symptoms with uveitis due to other causes. The objective of this study was to evaluate the leptospiral haemin-binding protein HbpA as a diagnostic antigen for the serodiagnosis of leptospiral uveitis. Serum samples from patients, clinically diagnosed with leptospiral uveitis, were tested by ELISA for anti-HbpA antibodies and compared against the 'gold standard' microscopic agglutination test (MAT). Non-leptospiral uveitis and normal healthy individuals were used as controls. A total of 60 serum samples from patients suffering from leptospiral uveitis were studied, obtained from Aravind Eye Hospital, Madurai. Anti-HbpA IgG antibodies were detected in 92 % of patients clinically diagnosed with leptospiral uveitis, indicating that it is more sensitive than MAT, which had a seropositivity of only 50 %, and better than the commercially available Pan Bio IgM ELISA (81 %). The mean anti-HbpA antibody titre was significantly higher in leptospiral uveitis patients compared with controls ($P<0.05$). The antigen showed negligible cross-reactivity with non-leptospiral uveitis samples and cataract controls. We conclude that HbpA IgG ELISA identified cases of uveitis with leptospirosis aetiology and proved to be useful in differentiating them from other forms of uveitis.

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INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic spirochaetal bacteria belonging to the genus *Leptospira*. It is more prevalent in tropical countries, where the humid conditions favour the spread and transmission of the disease. Rodents and other wild animals and domestic animals serve as reservoirs of infection and shed live leptospires in their urine and contaminate the immediate environment. Humans become accidental hosts as a result of occupational and recreational activities; they are infected either by direct contact with infected animals or indirectly via contaminated water or soil.

The disease is grossly underreported because of its broad range of clinical manifestations, which range from mild flu-like illness to the severe, often fatal form called Weil's disease. In the latter, fatality is often due to multiorgan failure including hepatorenal failure, myocarditis, severe pulmonary haemorrhage with respiratory distress and meningitis (Bharti *et al.*, 2003). Clinically, the disease presents with symptoms that mimic a number of other unrelated infections such as influenza, meningitis, hepatitis or dengue or viral haemorrhagic fevers. Uveitis is an important late complication (Rathinam, 2005) that occurs around 2–6 months after systemic disease. The onset and severity of leptospiral uveitis are quite variable, and the severity does not correlate with the severity of systemic disease. Common ocular signs include unilateral or bilateral, acute, non-granulomatous anterior or pan-uveitis, hypopyon, optic disc oedema, retinal vasculitis and membranous vitreous opacities (Rathinam, 2005). Unlike systemic leptospirosis, leptospiral uveitis carries good prognosis.

The diagnosis of leptospirosis is based primarily on either isolation of the infecting organism or demonstration of a rise in the levels of anti-leptospiral antibodies in the serum of the infected subject. Culture is difficult and is usually not done and, conventionally, serological testing by microscopic agglutination test (MAT) is accepted as the gold standard. The latter, however, is time-consuming and has several disadvantages; it requires paired sera, especially in endemic areas, maintenance of several pathogenic serovars and trained personnel to perform the test (Faine *et al.*, 1999). Hence, there is a need to develop a simple, easy-to-perform immunoassay for the detection of antibodies against pathogen-specific leptospiral antigens expressed *in vivo*. ELISAs based on whole-cell leptospiral antigen preparations (Terpstra *et al.*, 1985; McBride *et al.*, 2007) and recombinant outer-membrane proteins (Flannery *et al.*, 2001) including

Abbreviation: MAT, microscopic agglutination test.

LipL32 (Haake *et al.*, 2000; Smits, 2005), LipL41 and OmpL1 (Natarajaseenivasan *et al.*, 2008), Hsp58 (Park *et al.*, 1999), Lig proteins (Croda *et al.*, 2007), LruA and LruB (Verma *et al.*, 2008) have been developed in order to detect leptospiral infection in humans.

In a previous study, we identified a haemin-binding, TonB-dependent outer-membrane protein, HbpA (Sritharan *et al.*, 2005; Asuthkar *et al.*, 2007), in pathogenic *Leptospira* species. HbpA is an 81 kDa protein expressed upon limitation of iron. The mammalian host limits iron to an invading pathogen as part of its innate immune response (Kochan, 1977) and therefore the expression of the protein, as inferred from the presence of anti-HbpA antibodies, is not surprising (Sridhar *et al.*, 2008). We demonstrated the diagnostic potential of HbpA in screening for leptospirosis (Sridhar *et al.*, 2008; Sivakolundu *et al.*, 2011) and here we extend the usefulness of HbpA IgG ELISA to the serological diagnosis of clinically confirmed cases of leptospiral uveitis that were negative by MAT.

METHODS

Subjects. The human subjects included in this study are detailed in Table 1 and include patients (and contacts) attending the Uvea Clinic, Aravind Eye Hospital, Madurai.

Leptospiral strains. Leptospiral reference strains (Table 2) were obtained from the Royal Tropical Institute (KIT), Amsterdam, The Netherlands. They were maintained in semi-solid (0.15 % agar) and liquid EMJH medium.

MAT. All serum samples were screened by MAT using a panel of 20 serovars of *Leptospira* species (Table 2). The end point was taken as the highest dilution of the serum in which 50 % of the organisms were agglutinated or in which there was a 50 % reduction in the number of organisms compared with the control. Titres $\geqslant$1 : 100 were considered as positive (Terpstra *et al.*, 1985; Priya *et al.*, 2003).

Recombinant HbpA and anti-HbpA antibodies. Recombinant HbpA (rHbpA) and anti-HbpA antibodies were prepared as reported previously (Asuthkar *et al.*, 2007). The polyclonal antibodies were tested for cross-reactivity against whole-cell sonicates of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris*, *Klebsiella pneumoniae* (collection of the L. V. Prasad Eye Institute, Hyderabad, India) and *Mycobacterium tuberculosis* ATCC 27294. Aliquots (500 ng) of these cell-free sonicates were coated on polystyrene microtitre plates (Corning) and incubated with anti-HbpA antibodies (1 : 100 dilution) for 2 h, followed by the addition of goat anti-rabbit IgG alkaline phosphatase conjugate (1 : 5000 dilution; Bangalore Genei). The alkaline phosphatase substrate *p*-nitrophenyl phosphate (100 µl; Bio-Rad) was added and incubated for 15 min and the absorbance was read at 405 nm in an ELISA reader (model 680XR; Bio-Rad). Purified rHbpA (100 ng) and whole-cell sonicates of *Leptospira interrogans* serovar Manilae, grown under high- and low-iron conditions (Asuthkar *et al.*, 2007), were included as controls. The experiment was repeated three times with each reaction set up in duplicate.

HbpA IgG ELISA. Optimal antigen concentration and serum dilution were determined by chequerboard titration using a known positive serum sample, according to published protocols (Sivakolundu *et al.*, 2011). Briefly, flat-bottomed polystyrene microtitre plates (Corning) were coated with 250 ng rHbpA (100 µl in 0.1 M bicarbonate buffer, pH 9.2) for 3 h at 37 °C, followed by overnight incubation at 4 °C. The plates were washed three times with 10 mM PBST (0.01 M phosphate buffer, pH 7.4, with 0.05 % Tween 20 and 0.9 % NaCl) and

Table 1. Human subjects included in the study

Group	Cases (*n*)	Clinical criteria for inclusion	Remarks
I	60	Subjects were clinically confirmed cases of leptospiral uveitis. Inclusion criteria were acute, non-granulomatous, anterior or pan-uveitis. Patients were subjected to detailed clinical history, extensive review of systems, complete ophthalmic examination by slit lamp and indirect ophthalmoscopy. All routine laboratory and ancillary tests were done. All other causes of uveitis were ruled out. Based on the results of MAT, 30 seropositive and 30 seronegative cases were included	Previous leptospiral infection was strongly indicated in patients with hypopyon, disc oedema, vasculitis and vitreous membrane or vitreous exudates (Priya et al., 2003, 2007)
II	20	Clinically diagnosed cases of systemic leptospirosis according to WHO criteria (Rathinam, 2005), seropositive by MAT	Patients were referred from nearby hospitals for confirmation of leptospirosis by serodiagnosis
III	20	Non-leptospiral uveitis controls. Ocular inflammation due to other causes; all were seronegative by MAT*	No evidence of fever within the recent past of attending the Uvea Clinic
IV	10	Cataract controls. No ocular inflammation and all were seronegative by MAT	
V	20	Endemic controls. Healthy normal individuals with no evidence of disease and MAT-negative	

*This group includes patients with Vogt–Koyanagi–Harada syndrome (4), posterior choroiditis (1), Behcet's uveitis (3), sympathetic ophthalmia (2), unilateral granulomatous pan-uveitis with tractional retinal detachment (1), sarcoid uveitis (1), Fuch's heterochromic uveitis (6), bilateral granulomatous uveitis (1) and HLA B27-related uveitis (1).

Table 2. Panel of reference strains of *Leptospira* used in MAT

Strain no.	Serogroup	Serovar	Strain
1	Australis	Australis	Ballico
2	Autumnalis	Autumnalis	Akiyami A
3	Icterohaemorrhagiae	Copenhageni	Wijnberg
4.	Louisiana	Lanka	R 740
5	Semaranga	Patoc	Patoc I
6	Australis	Bratislava	Jez Bratislava
7	Sejroe	Hardjo	Hardjoprajitno
8	Pomona	Pomona	Pomona
9	Pyrogenes	Alexis	HS616
10	Icterohaemorrhagiae	Lai	Lai
11	Sejroe	Wolffi	3705
12	Australis	Bharathy	–*
13	Javanica	Menoni	Kerala
14	Celledoni	Celledoni	Celledoni
15	Louisiana	Louisiana	LSU1945
16	Autumnalis	Bulgarica	Nikolaevo
17	Cynopteri	Cynopteri	3522C
18	Australis	Lora	Lora
19	Grippotyphosa	Ratnapura	Wumalasena
20	Andamana	Andamana	CH11

*Clinical isolate (Kuriakose *et al.*, 1997).

then blocked with 5 % (w/v) BSA in PBST for 2 h at 37 °C, and then 100 µl human serum sample (1 : 200 dilution) was added and incubated for 2 h at 37 °C. After washing, the plates were incubated for 2 h at 37 °C with 100 µl rabbit anti-human IgG alkaline phosphatase conjugate (1 : 5000 dilution; Bangalore Genei). Substrate was added and the absorbance was read as described above. Antigen and antibody blanks were included in the test to check for background absorbance. A known positive serum sample and a serum sample from a healthy individual were used as positive and negative controls, respectively.

Commercial Pan Bio IgM ELISA. All the test serum samples were tested for anti-leptospiral antibodies with the commercial Pan Bio Leptospira IgM ELISA kit (Inverness Medical Innovations Australia). Serum (100 µl, diluted 1 : 100) was added to the pre-coated microwell strip. Plates were incubated at 37 °C for 30 min and then washed and 100 µl peroxidase-conjugated anti-human IgM was added, followed by incubation for 30 min at 37 °C. After sufficient washes to remove the unbound conjugate, 100 µl tetramethyl benzidine/hydrogen peroxide was added and the plates were incubated for 10 min. The reaction was stopped by the addition of 100 µl 1 M phosphoric acid and the absorbance was read at 450/600 nm using an ELISA reader (model 680XR; Bio-Rad). The cut-off value was calculated by multiplying the mean absorbance of the calibrator sample replicates with the calibration factor (according to the manufacturer's instructions). The Pan Bio unit for each sample was calculated by dividing the absorbance of the sample by the cut-off value and then multiplying by 10. A score <9 units indicated a negative result, 9–11 units an equivocal result and >11 units a positive result, indicating the presence of leptospira-specific IgM antibodies.

Statistical analysis. Data were analysed using the SPSS version 13 statistical package (SPSS Inc.). The non-parametric Kruskal–Wallis test was used to assess differences in HbpA antibody levels between patients and controls; $P<0.05$ was considered significant. The relative sensitivity and specificity of ELISA for serodiagnosis of leptospirosis were evaluated as follows. Sensitivity (%) $=[a/(a+c)]\times 100$, where a is the number of true-positive samples and c is the number of false-negative samples. Specificity (%) $=[d/(b+d)]\times 100$, where d is the number of true-negative samples and b is the number of false-positive samples.

RESULTS

MAT

All the serum samples included in the study were screened for anti-leptospiral antibodies by MAT; 50 % of the leptospiral uveitis cases were negative by MAT. Icterohaemorrhagiae was identified to be the predominant infecting serogroup in both systemic leptospirosis (30 %) and leptospiral uveitis (35 %) patients. The other major infecting serogroups were Autumnalis, Australis, Louisiana and Pyrogenes (Table 3). Groups III, IV and V, representing non-leptospiral uveitis, cataract and normal healthy controls, tested negative by MAT.

HbpA IgG ELISA

Characterization of anti-HbpA antibodies Analysis of polyclonal HbpA antibodies for cross-reactivity against other bacterial whole-cell sonicates revealed low levels of reactivity ($A_{405}<0.2$). A threefold increase in the absorbance was observed with the sonicate from *L. interrogans* serovar Manilae grown under low-iron conditions (A_{405} 0.633 ± 0.016) in comparison with high-iron conditions (A_{405} 0.229 ± 0.013), with maximal reactivity (A_{405} 2.380 ± 0.08) observed with purified rHbpA protein (Fig. 1).

HbpA IgG ELISA Based on the mean absorbance plus two standard deviations of the group V healthy controls, the cut-off value was established as 0.376. A significant increase in antibody levels was observed in the serum of group I leptospiral uveitis patients compared with controls ($P<0.05$) in both MAT-positive (93 % positive) and MAT-negative (90 %) cases included in group I (Fig. 2, Table 4). Similarly, the levels of antibodies among systemic leptospirosis patients were also significantly higher compared with controls ($P<0.05$). Low levels of antibodies above the cut-off value were identified in three non-leptospiral uveitis patients and three cataract controls. Evaluation of these results with MAT and clinical diagnosis as the reference, revealed 93 and 92 % sensitivity and 58 and 86 % specificity, respectively (Tables 4, 5 and 6).

Pan Bio IgM ELISA

Among the leptospiral uveitis patients in group I, 90 % of MAT-positive and 73 % of MAT-negative samples were positive by IgM ELISA. Forty per cent of non-leptospiral uveitis cases and cataract controls and 15 % of healthy controls were also positive. Evaluation of these results with MAT and clinical diagnosis as the reference revealed 90 and 82 % sensitivity and 54 and 70 % specificity, respectively (Table 4, 5 and 6).

Table 3. Reactive serogroups identified by MAT in patients with leptospiral uveitis and systemic leptospirosis

Strain no.	Reactive serogroup	Positive cases (*n*)	
		Leptospiral uveitis	Systemic leptospirosis
1	Australis	6	3
2	Autumnalis	7	3
3	Icterohaemorrhagiae	7	6
4	Louisiana	2	1
5	Semaranga	0	3
6	Sejroe	2	0
7	Pomona	1	0
8	Pyrogenes	5	2
9	Javanica	0	1
10	Andamana	0	1
Totals		30	20

DISCUSSION

Uveitis refers to inflammation of the uvea or the middle layer of the eye. The aetiology of the disease is multifold and, among the infectious agents, pathogenic leptospires contribute to ocular inflammation in humans (Rathinam, 2005). Ocular manifestations are commonly seen in leptospiral infections. Of the individual ocular signs, the combination of acute, non-granulomatous pan-uveitis, hypopyon, vasculitis, optic disc oedema, membranous vitreous opacities and absence of choroiditis or retinitis has a high predictive value for the clinical diagnosis of leptospiral uveitis. There is a need for a diagnostic test to distinguish leptospiral uveitis from other types of uveitis. In this study, we report the diagnostic potential of the haemin-binding protein HbpA in the identification of leptospiral uveitis. This study was done on patients attending the Uvea Clinic in Aravind Eye Hospitals, Madurai, one of the leading eye hospitals in India.

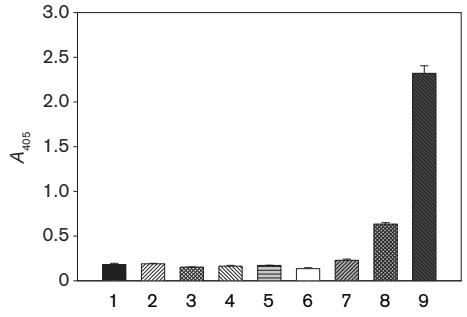

Fig. 1. ELISA to detect cross-reactivity of anti-HbpA antibodies with whole-cell sonicates of other bacteria: 1, *Proteus vulgaris*; 2, *Escherichia coli*; 3, *Pseudomonas aeruginosa*; 4, *Klebsiella pneumoniae*; 5, *Mycobacterium tuberculosis*; 6, *Staphylococcus aureus*; 7, *Leptospira interrogans* serovar Manilae, high-iron conditions; 8, *L. interrogans* serovar Manilae, low-iron conditions; 9, purified rHbpA protein. The plate was coated with whole-cell sonicates of different bacteria and ELISA was performed as described in Methods. Error bars represent the standard deviation of the mean from two independent experiments set up in duplicate.

HbpA was first reported as an iron-regulated, haemin-binding protein in serovar Lai (Sritharan *et al.*, 2005; Asuthkar *et al.*, 2007). Its absence from non-pathogenic *Leptospira* species and the *in vivo* expression of the protein, coupled to its usefulness in screening of clinical isolates by PCR (Sridhar *et al.*, 2008) and ELISA (Sivakolundu *et al.*, 2011), led us to extend its usefulness to the diagnosis of leptospiral uveitis. Recombinant HbpA was prepared as described previously (Asuthkar *et al.*, 2007) and we showed that there are no epitopes in the protein that are shared with other bacterial proteins. There was low cross-reactivity with other TonB-dependent proteins within the same organism, as reflected by the low reactivity of the leptospiral extract of *L. interrogans* serovar Manilae grown under high-iron conditions; a threefold increase was seen in extracts of low-iron-grown organisms as a result of increased expression of HbpA.

As this region of India is endemic for the disease, a baseline titre was determined for ELISA-based detection of IgG antibodies against HbpA and a cut-off was established from the data obtained from normal healthy controls (group V). The endemic nature of the disease accounts for the values observed in groups III, IV and V. Sera from all five groups were tested by the conventional 'gold standard' MAT. The predominant infecting serogroups (Icterohaemorrhagiae, Australis and Autumnalis) in MAT-positive leptospiral uveitis (group I) were similar to those reported for systemic leptospirosis patients, in agreement with our earlier reports (Priya *et al.*, 2003, 2007). Patients with systemic leptospirosis (group II), all of whom were MAT-positive, showed high levels of anti-HbpA IgG antibodies, as expected. Interestingly, 92 % of the patients clinically identified as having leptospiral uveitis (group I) showed significantly high levels of anti-HbpA antibodies (Fig. 2) compared with controls ($P<0.05$), though 50 % of them

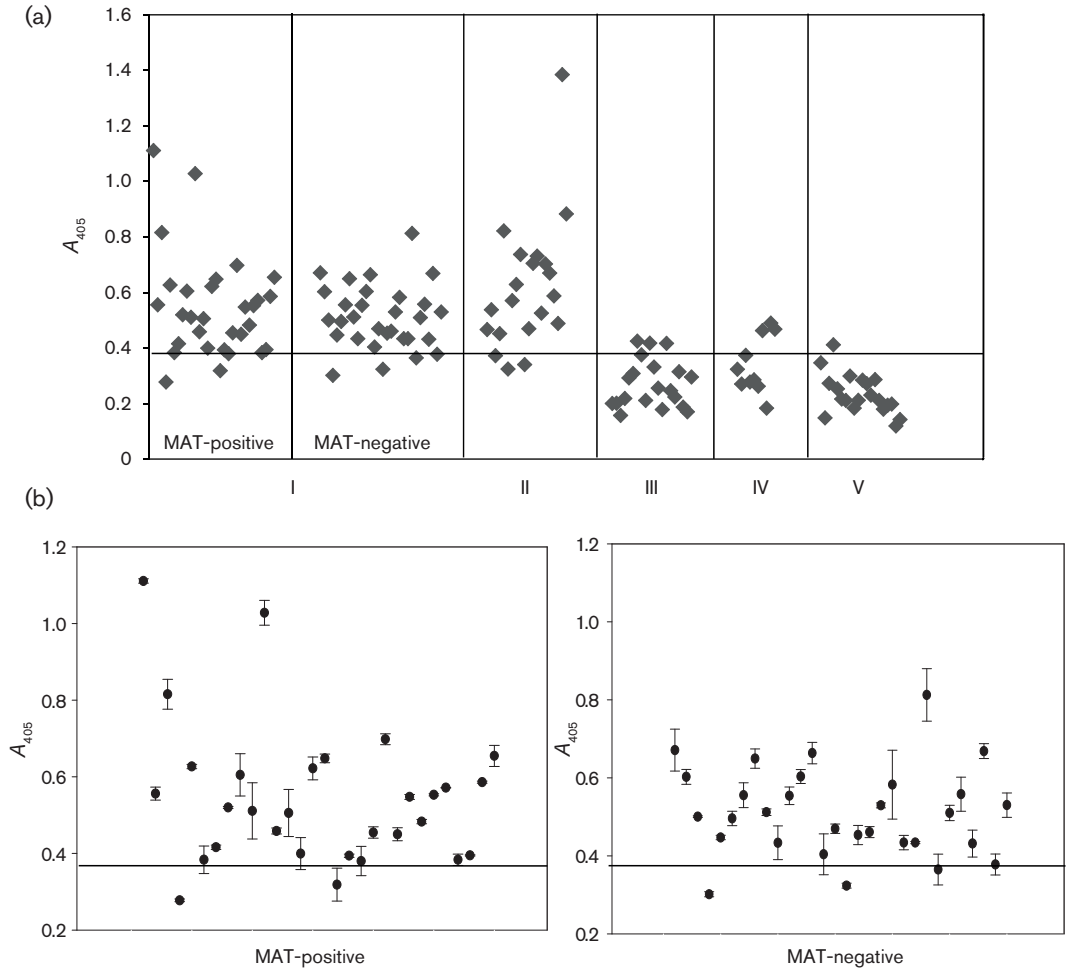

Fig. 2. Serum levels of anti-HbpA antibodies in patients with leptospiral uveitis. (a) Level of anti-HbpA antibodies in the serum of patients with leptospiral uveitis (group I) compared with non-leptospiral uveitis (group III), cataract cases (group IV), normal healthy controls (group V) and patients with systemic leptospirosis (group II). The values obtained in different patient groups are shown in a scatter plot. The non-parametric Kruskal–Wallis test showed significant differences between patient and control groups ($P<0.05$). (b) Almost identical levels of antibodies observed in both MAT-positive (left) and -negative (right) cases of leptospiral uveitis. The horizontal lines in the figure represent the cut-off for the ELISA, which was calculated using healthy endemic controls.

were found to be MAT-negative. The failure of MAT to identify all clinically proven cases of leptospiral uveitis, as seen in this study and in an earlier study (Priya *et al.*, 2003), both showing a low specificity of 58 %, indicates strongly that MAT cannot be used as the gold standard for diagnosis of leptospiral uveitis, as is currently being done

Table 4. Comparison of HbpA IgG ELISA and Pan Bio IgM ELISA in serodiagnosis of leptospiral uveitis

The number of positive cases/cases tested identified by HbpA IgG ELISA and Pan Bio IgM ELISA is shown, with percentages in parentheses.

ELISA	Leptospiral uveitis		Controls		
	MAT-positive	MAT-negative	Non-leptospiral uveitis (group III)	Cataract (group IV)	Normal controls (group V)
HbpA IgG	28/30 (93)	27/30 (90)	3/20 (15)	3/10 (30)	1/20 (5)
Pan Bio IgM	27/30 (90)	22/30 (73)	8/20 (40)	4/10 (40)	3/20 (15)

Table 5. Evaluation of ELISA-based detection of leptospiral uveitis compared with MAT and clinical diagnosis

ELISA	MAT			Clinical diagnosis		
	Positive	Negative	Total	Positive	Negative	Total
HbpA IgG						
Positive	28	34	62	55	7	62
Negative	2	46	48	5	43	48
Total	30	80	110	60	50	110
Pan Bio IgM						
Positive	27	37	64	49	15	64
Negative	3	43	46	11	35	46
Total	30	80	110	60	50	110

for systemic leptospirosis. There are also reports of the low sensitivity of MAT in diagnosing the latter, even at the optimal stage of systemic leptospiral infection (Limmathurotsakul *et al.*, 2012; Goris *et al.*, 2012). Agglutination of live organisms in MAT is due predominantly to the presence of antibodies against the highly antigenic lipopolysaccharide determinants that are specific to each serovar. In fact, this was the basis of the older classification of *Leptospira* into serogroups and serovars, based on their antigen-relatedness (Bharti *et al.*, 2003). Thus, the failure to detect those serovars prevalent in a particular geographical region can be attributed to their omission from the reference panel of live organisms used for the test. This could therefore account for the MAT-negativity in group I in this study; low levels of leptospira-specific antibodies at the late convalescent phase could also account for the failure to detect by MAT.

The failure of MAT to identify more than 50 % of clinically positive cases of suspected leptospiral uveitis led us to confirm the leptospiral aetiology using the commercial Pan Bio IgM ELISA. The MAT-negative patients identified clinically as patients with leptospiral aetiology because of the presence of pathognomonic clinical signs specific for leptospiral uveitis were positive both by HbpA IgG ELISA and the commercial Pan Bio IgM ELISA (Table 4). While both the ELISAs were better than MAT in the diagnosis of leptospiral uveitis, a comparative analysis of HbpA IgG ELISA and IgM ELISA (Tables 5 and 6) showed the better performance of HbpA IgG ELISA over IgM ELISA when compared with clinical diagnosis, with a specificity of 86 and 70 %, respectively. Though IgM ELISA has better potential for screening systemic leptospirosis (Winslow *et al.*, 1997; Bajani *et al.*, 2003), it has limited application in the diagnosis of leptospiral uveitis in this geographical region.

Table 6. Sensitivity and specificity of HbpA IgG and Pan Bio ELISAs

Criterion	MAT		Clinical diagnosis	
	HbpA IgG	Pan Bio IgM	HbpA IgG	Pan Bio IgM
Sensitivity (%)	93	90	92	82
Specificity (%)	58	54	86	70

The observations in this study have strengthened the diagnostic potential of HbpA for the screening of leptospirosis. The *in vivo* expression of HbpA and its presence only in pathogenic *Leptospira* species make it an ideal diagnostic antigen. The upregulation of HbpA by iron limitation and rise in temperature (Asuthkar *et al.*, 2007), encountered within the mammalian host, would therefore reflect active infection. In this study, HbpA IgG ELISA proved to be useful in the screening of cases of not only systemic leptospirosis but also leptospiral uveitis. The simple format of ELISA would be suitable for use in routine laboratories and would be cost-effective when compared with MAT and PCR. ELISA is a better alternative to MAT as it does not require live pathogenic cultures, can achieve high sensitivity and specificity and can be semi-automated. The results can be interpreted objectively and the analysis can be done in small laboratories without the need for any specialized equipment. The assay is rapid and the antigen-coated plates can be stored and used at any time of the year, unlike MAT, where the organisms have to be grown and monitored continuously so that they are in exponential phase for analysis. MAT may be useful in epidemiological studies for identifying the predominant serovar, but timely diagnosis of the leptospiral aetiology by HbpA IgG ELISA, irrespective of the infecting serovar, followed by the timely treatment of the disease will contribute to better control measures. Secondly, HbpA IgG ELISA will be simpler to use and more economical than *hbpA* PCR. Earlier, using primers specific for *hbpA* in the genome of serovar Lai, we identified all the serovars belonging to *L. interrogans* by PCR. While specific primers can now be designed from the increasingly available whole genome sequence data in order to identify other species (Bulach *et al.*, 2006), PCR may not be economically viable as a routine diagnostic test and, because of the inherent problem of false positivity, cannot be used as the sole diagnostic test. HbpA IgG ELISA therefore offers considerable promise for the screening of both systemic leptospirosis and leptospiral uveitis.

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