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## Insights into Leptospirosis, a Neglected Disease

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### 1. Introduction

Leptospirosis is a zoonotic disease affecting man and animals worldwide. The causative organisms are the pathogenic members of the spirochetes belonging to the genus *Leptospira* [Bharti et al., 2003; Levett, 2001]. The disease, more prevalent in tropical regions, especially in developing and under-developed countries has remained largely unnoticed due to poor diagnosis. This zoonotic disease is predominantly an occupational disease affecting farmers and others working in close association with animals. It has also been reported in events like water sports, swimming and other recreational activities. In the past decade there were several outbreaks of the disease occurring globally after natural calamities like floods and cyclones. Leptospirosis occurs both in rural and urban environments with wet and humid conditions favouring its transmission. Humans are accidental hosts whereas wild, domestic and peri-domestic animals serve as reservoir hosts with rodents playing a major role in the transmission of the disease. The infected animals shed the organisms via urine into the immediate environment and humans acquire the infection, either directly from the animals or indirectly from the contaminated environment.

In 1886, Adolf Weil first reported leptospirosis (see Box 1 for a historical perspective of the disease) as a syndrome characterized by splenomegaly, jaundice and nephritis that is now commonly reported as Weil's disease and is synonymous with leptospirosis [Levett, 2001]. In several countries in the Southeast Asia region, the disease is endemic but is grossly under-reported as the protean clinical manifestations are difficult to differentiate from other diseases like influenza (commonly called 'flu'), dengue and malaria and due to the lack of simple, rapid and efficient tests for early diagnosis. The need of the hour is a better understanding of host-pathogen interactions; unravelling the complex mechanisms elaborated by the pathogen to establish infection and the diverse efforts of the mammalian host to contain the pathogen and prevent disease development will aid in the identification of potential vaccine and diagnostic candidates. This is now possible with significant advances in the field of leptospirosis, especially the sequencing of several leptospiral genomes providing better insights into the biology of the causative organisms and the development of better genetic tools for *Leptospira* spp.

### Box 1: *Leptospira* and leptospirosis: a historical reflection

Several years after the first clinical reporting of the disease by Adolf Weil (1886), Inada and Ido (1915) demonstrated leptospire as the causative organisms for this disease [Inada et al., 1995]. Unfortunately, the sentinel observations made by Stimson [Stimson, 1907] went unrecognized. He demonstrated the presence of spirochetes in renal tubule specimens from a patient diagnosed to have died of yellow fever and called them *Spirocheta interrogans*, as the hook at the ends resembles a question mark. The free-living or the saprophytic counterparts of these organisms were found in fresh water and were called as *Spirocheta biflexa* [Walbach and Binger, 1914]. Ido and his group (1917) unequivocally proved the role of rats in the transmission of the disease to humans that led to the establishment of leptospirosis as a zoonotic disease. The disease came to be recognised as an occupational disease and was referred to as 'akiyami' the harvest fever in Japan, cane cutter's disease in Australia, rice field leptospirosis in Indonesia and so on. The disease was reported in India, as early as 1903 by Chowdry (as reported by [Vijayachari et al., 2008]). An extensive and detailed account of the disease was documented by Taylor & Goyle [Taylor and Goyle, 1931] in the Andaman archipelago. Today, the leptospiral etiology of the 'Andaman fever' or 'Andaman haemorrhagic fever' (AHF) accounting for several deaths in the Andaman & Nicobar Islands, India is well known. The contribution of Faine and his group in the understanding of the basic biology of these organisms, identification of multitude of serovars and the range of mammalian hosts, including humans, wild and domestic animals contributed significantly to the field of leptospirosis. Subsequent years focused on leptospiral proteins, including LipL41 & Omp1 [Haake et al., 1993; Haake et al., 1999], LipL32 [Haake et al., 2000], LigA & LigB proteins [Koizumi and Watanabe, 2004; Matsunaga et al., 2003], HbpA [Sritharan et al., 2005], [Asuthkar, 2007]. Ren et al [Ren et al., 2003] was the first to sequence the genome of the pathogenic *L. interrogans* serovar Lai to be followed by others deciphering the genome of Copenhageni [Nascimento et al., 2004], two strains of serovar Hardjo belonging to *L. borgpetersenii* [Bulach et al., 2006] and the non-pathogenic *L. biflexa* serovar Patoc [Picardeau et al., 2008]. Today we have before us a vast amount of information on these organisms that needs to be mined towards development of better control measures.

## 2. Biology of leptospire

### 2.1 General features

Leptospire are obligate aerobic spirochetes with characteristic hooks at their ends. They are thin (0.25  $\mu\text{m}$ ) and long (6-25  $\mu\text{m}$ ) and can easily pass through 0.2  $\mu\text{m}$  filters. These highly motile organisms showing the characteristic cork-screw movement include the pathogenic and saprophytic members (see Box 2 for classification); the former are usually shorter than the non-pathogenic members.

As these organisms are too thin, it is difficult to visualise them by the conventional stains, though occasionally silver staining is used. The characteristic cork-screw movement of these organisms in thin wet preparations makes it possible to view these organisms by dark field and phase-contrast microscopy.

**Box 2: Classification of *Leptospira* spp.**

Taxonomically, *Leptospira* are classified as Gracillicutes (Division), Scotobacteria (Class), Spirochaetales (Order), *Leptospiraceae* (Family) and *Leptospira* (Genus) [Paster and Dewhirst, 2000]. In addition to the genus *Leptospira*, the genera *Leptonema* and *Turneria* are included in the family *Leptospiraceae*. The different members of the genus *Leptospira* are classified based on their antigen-relatedness (serological) or DNA-relatedness (molecular) as detailed below:

- **Serological classification:** Earlier classification, still used in epidemiological studies was based on antigen-relatedness. Broadly, pathogenic *Leptospira* were grouped as belonging to *Leptospira interrogans* and the non-pathogenic as *Leptospira biflexa*. The former includes more than 200 serovars, classified on the basis of structural heterogeneity in the carbohydrate component of the lipopolysaccharide [Bharti et al., 2003; Levett, 2001; Vijayachari et al., 2008] that resulted in the generation of specific antibodies in the mammalian host. The non-pathogenic *L. biflexa* consists of about 60 serovars. The newly added members include the serovars Sichvan (serogroup Sichvan), Hurstbridge (serogroup Hurstbridge) and Portblairi (serogroup Sehgal) [Brenner et al., 1999; Vijayachari et al., 2004].
- **Molecular / genotypic classification:** The current classification is based on DNA-relatedness. In the Subcommittee on the Taxonomy of *Leptospiraceae* held in Quito, Ecuador in 2007, it was decided to group them as species [Adler and Moctezuma, 2010]. The saprophytic species include *Leptospira biflexa*, *Leptospira wolbachii*, *Leptospira kmetyi*, *Leptospira meyeri*, *Leptospira vanthielii*, *Leptospira terpstrae* and *Leptospira yanagawae*. The pathogenic species include *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira noguchii*, *Leptospira weilii*, *Leptospira alexanderi* and *Leptospira alstoni*. An additional group comprising of *Leptospira inadai*, *Leptospira broomii*, *Leptospira fainei*, *Leptospira wolffii* and *Leptospira licerasiae* consisted of species of unclear pathogenicity [Ko et al., 2009].

Molecular classification allows for the clear identification of distinct subtypes. For example, Hardjoprajitno and Hardjobovis, grouped earlier under serovar Hardjo now belong to *L. interrogans* and *L. borgpetersenii*.

\*Historically, the first leptospiral strain reported by Inada and Ido (1915) was called as *Spirochaeta Icterohaemorrhagiae japonica*.

**2.2 Growth and culture conditions**

Leptospire grow relatively slowly with a doubling time of approximately 6 - 8 hours. *In vitro* cultures are usually maintained at temperatures between 28° - 30°C. They have simple, but unique nutritional requirements. They utilize long-chain fatty acids as the carbon and energy source and require vitamins B1 & B12 as growth factors. Tween 80 is commonly used in artificial culture media as the source of carbon and it is necessary to include bovine serum albumin (BSA) to bind the free fatty acids that are toxic to these organisms. 10% rabbit serum is added in certain media, as a rich source of vitamins and albumin promotes the growth of these organisms [Ellis and Michno, 1976; Johnson and Gary, 1963a, b]. The conventional media for growing leptospire include the bovine serum albumin-based Ellinghausen-McCullough-Johnson-Harris (EMJH) medium [Ellinghausen and McCullough, 1965] and serum-containing Korthof's and Fletcher's media. Liquid media is used for

propagating the organisms and long-term maintenance is done in semi-solid medium containing 0.1 - 0.2% agar, in which the organisms grow sub-surface and appear as an opaque mass called the 'Dinger's ring'. *Leptospira* are not affected by 5-fluorouracil and thus media can be made selective for the growth of leptospire from clinical specimens by the addition of 5-fluorouracil and neomycin sulphate [Ellis and Michno, 1976].

### 2.3 Membrane architecture and membrane proteins

*Leptospira* exhibits a surface architecture that resembles Gram-negative bacteria and consists of the outer membrane, the periplasmic region and the inner cytoplasmic membrane (Fig. 1). The peptidoglycan layer however is closely associated with the cytoplasmic membrane, unlike in other Gram-negative bacteria where it is located close to the outer membrane [Adler and Moctezuma, 2010]. The two internal flagella (endoflagella), arising from each

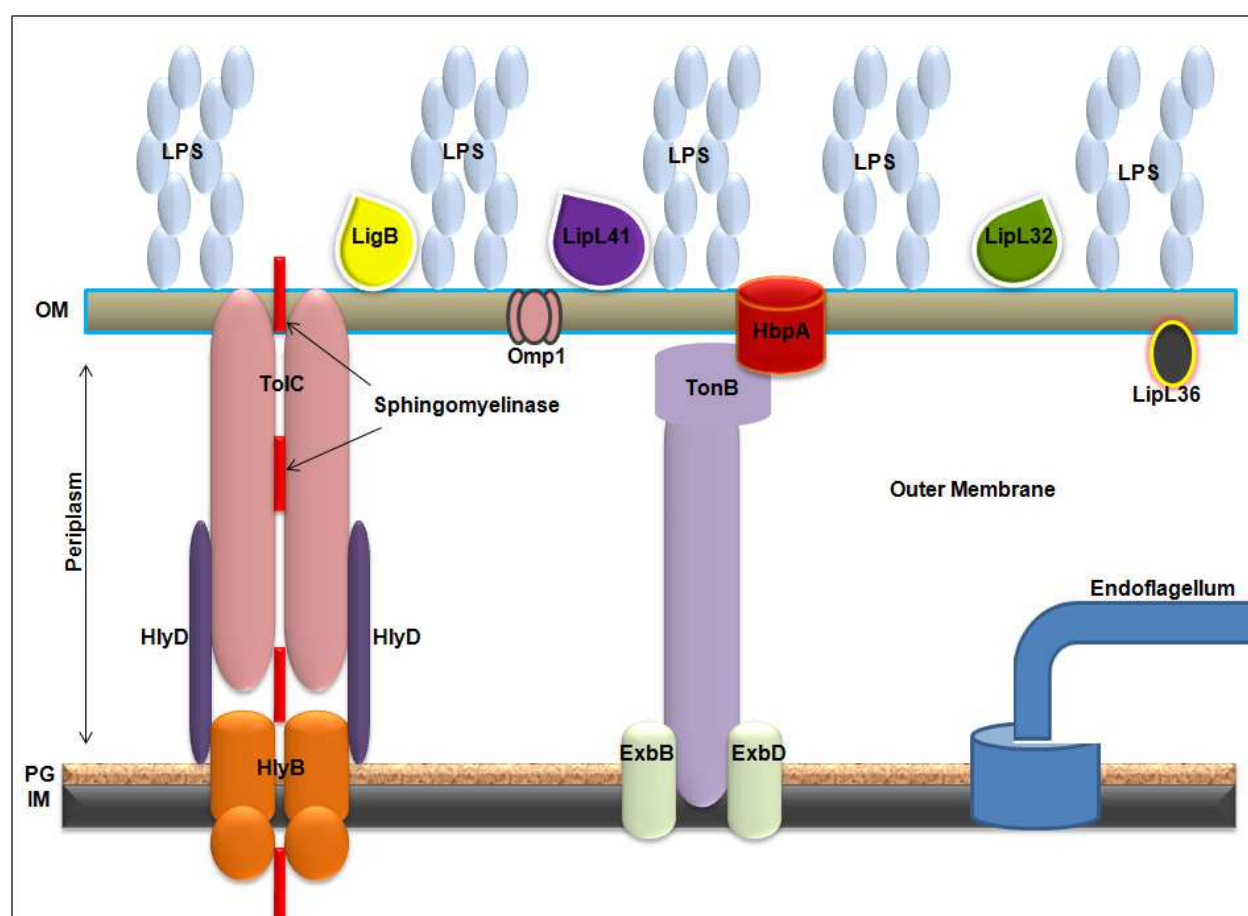


Fig. 1. Leptospiral membrane architecture and distribution of membrane proteins. The outer membrane (OM) with the associated lipopolysaccharide (LPS) and outer membrane proteins, including the lipoproteins LipL32, LipL41 and LigB protein are in contact with the immediate environment. The hemin-transporter HbpA is an example of a TonB-dependant outer membrane receptor that mediates the transport of hemin via the TonB protein located in the inner membrane (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. The peptidoglycan (PG) is associated with IM.



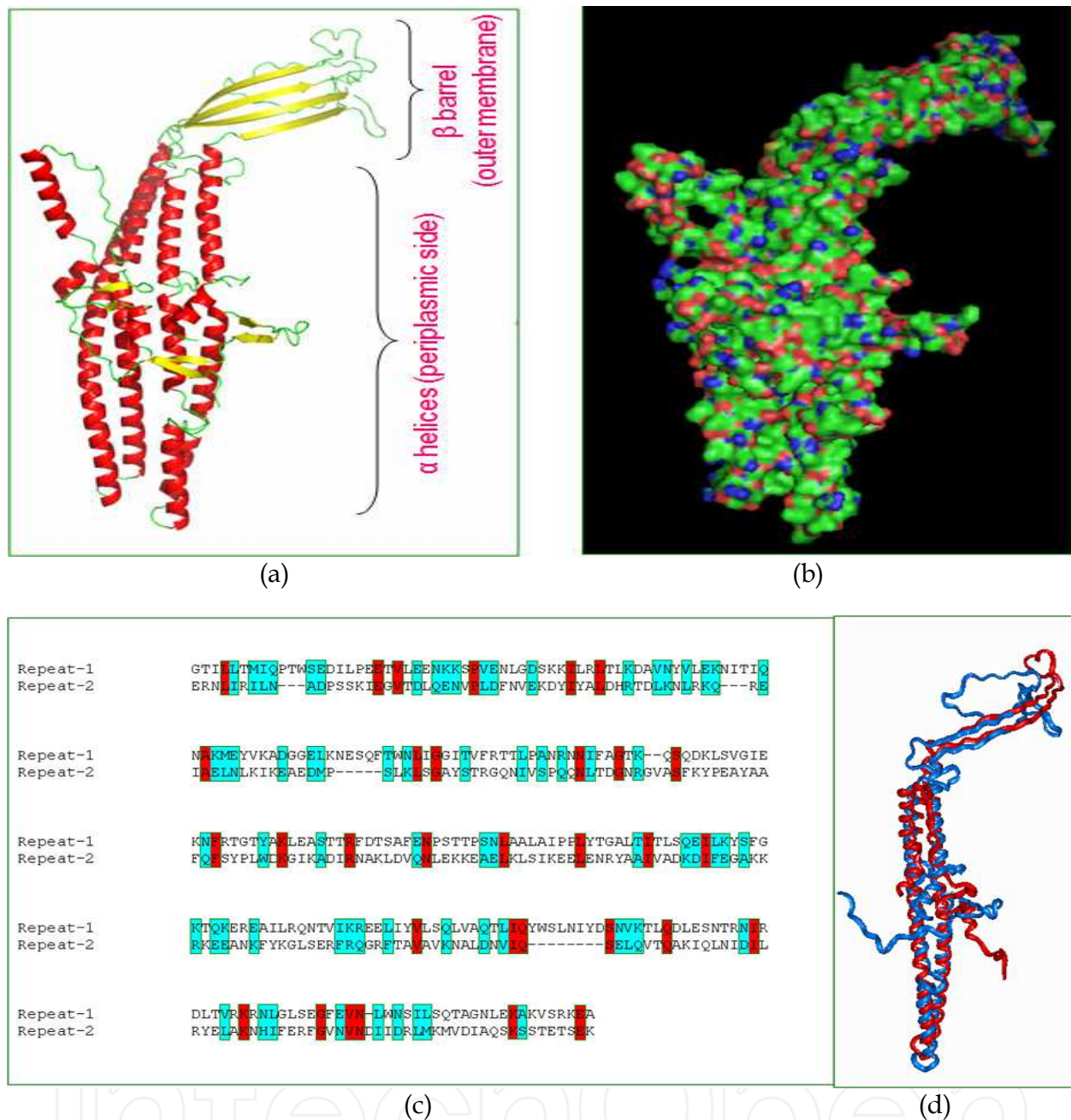
end of the spirochete and extending towards the centre [Hovind-Hougen, 1976; Li et al., 2000] are responsible for the cork-screw movement of these organisms. The lipopolysaccharides (LPS), located within the outer membrane are highly antigenic and their structural variations give rise to the large diversity of the serovars and serotypes seen among the leptospiral species. Haake and his group [Haake et al., 1993; Haake et al., 2000; Haake et al., 1998; Haake and Matsunaga, 2002, 2005; Haake et al., 1999; Haake et al., 2004; Haake et al., 1991; Lo et al., 2006] contributed significantly to the identification and isolation of the leptospiral membrane proteins. They used different isolation techniques and classified the outer membrane proteins into transmembrane, lipoprotein and peripheral membrane proteins. The porin OmpL1, the first leptospiral OMP to be described [Haake et al., 1993] is a transmembrane protein present as a trimer. The second class of leptospiral OMPs, the lipoproteins, constitute the most abundant of the leptospiral proteins in the outer membrane, to which they are anchored by fatty acids.

The leptospiral lipoproteins include LipL32 (also called as hemolysis-associated protein-1; Hap-1), LipL36, LipL41, LipL31, LipL21, LipL45 and LipL48. LipL31 is located on the inner membrane whereas LipL21 [Cullen et al., 2002; Cullen et al., 2003; Cullen et al., 2005], LipL32 [Haake et al., 2000], LipL41, LipL36 [Haake et al., 1998], LipL45 [Matsunaga et al., 2002] and LipL48 are found in the outer membrane. Some of these proteins, namely LipL32 and LipL41 are conserved among many pathogenic *Leptospira* serovars and are also expressed during infection. Among the third class of proteins, classified as peripheral membrane proteins, the protein P31<sub>LipL45</sub> can be released from membranes by urea and can be partitioned into both Triton X - 114 detergent and aqueous phases [Haake et al., 2000; Haake and Matsunaga, 2002].

Other proteins that need mention here 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 iron and vitamin B12. Our search for iron transporters led us to the identification of the TonB-dependant hemin-binding protein HbpA in the pathogenic *L. interrogans* serovar Lai [Asuthkar, 2007; Sritharan et al., 2005]. It is an iron-regulated hemin-binding protein with the characteristic  $\beta$  - barrel structure composed of 22 anti-parallel  $\beta$ -sheets and a globular N-terminal region. This cell surface protein, expressed in low iron organisms mediates the transport of iron (discussed in detail in Section). TolC proteins are bacterial efflux proteins; in *E. coli* they are associated with the transport of  $\alpha$ -hemolysin [Thanabalu et al., 1998]. A 63 kDa protein of *L. interrogans* serovar Lai, partitioning into the Triton X-114 detergent phase [Velineni et al., 2009] proved to be TolC efflux protein encoded by LA0957. The three-dimensional structure of the protein is identical to the corresponding homologue in *E. coli*. It is a trimeric molecule with each monomer consisting of 4 strands with  $\beta$  barrel and 4  $\alpha$  helices (Fig. 2a & b). There are two structural repeats (Fig. 2c), namely repeat 1 consisting of amino acid residues 16 - 294 and repeat 2 consisting of amino acid residues 295 - 557 respectively. The sequence and structures of the N and C terminal halves of the protein monomer are thus identical, as revealed by the alignment of these repeats (Fig. 2d).

## 2.4 Organization of the leptospiral genome

Whole genome sequencing of different leptospiral species is a major contribution to the advancement of knowledge about these organisms. The genome sequence of *L. interrogans*



([Velineni et al., 2009], Reproduced with permission from Online J Bioinformatics)

Fig. 2. Structural organization of the efflux protein TolC in serovar Lai. Panel (a) shows the folding of the monomeric unit of TolC, with the  $\beta$  barrel and 4  $\alpha$  helices positioned in the outer membrane and periplasmic regions. Panel (b) shows the charge distribution on the surface of the molecule, with red, blue and green regions representing the electronegative, electropositive and the non polar residues respectively. Panels (c) and (d) show the two structural repeats in the molecule and the superimposed secondary structure of these structural repeats respectively.

serogroup Icterohaemorrhagiae serovar Lai strain 56601 was first made available in 2003 [Ren et al., 2003] closely followed by that of serovar Copenhageni strain Fiocruz L1-130 belonging to the same species [Nascimento et al., 2004]. Both these genomes are larger and

differ considerably from those of the related spirochetes, *Treponema pallidum* and *Borrelia burgdorferi*, indicating their divergence from the phylum. Today, two additional genome sequences, namely that of *L. borgpetersenii* serovar Hardjo strains L550, JB197 [Bulach et al., 2006] and *L. biflexa* serovar Patoc strain Patoc 1 (Ames strain) [Picardeau et al., 2008] are available

With the exception of the non-pathogenic *L. biflexa* with three chromosomes, the others have genomes that consist of two chromosomes, the larger chromosome CI (approximately 4 Mb) and the relatively smaller chromosome CII (300 Kb). The non-pathogenic *L. biflexa* also shows the presence of the 74 kb leptospiral bacteriophage LE1 [Bourhy et al., 2005]. Re-annotation [Bulach et al., 2006] in both the serovars Lai and Copenhagenii has now made the number of recognized CDS to be 3613 and 3530 respectively. *Leptospira borgpetersenii* genome is 16% (approximately 700 Kb) smaller than *L. interrogans* and the reduction in the CDS (3166) is due to insertion sequence-mediated genome reduction.

Comparative genome analysis [Ko et al., 2009] provides insights into the role of specific gene products that enable the organisms to survive in their natural environment. For eg., the genome of *L. biflexa*, a free-living organism with 3590 CDS uses the additional genes to thrive in aquatic environments. Among the two pathogenic species, *L. borgpetersenii* can survive only within the mammalian host as against the additional advantage of survival of *L. interrogans* in wet and moist environments outside the mammalian host. Thus the higher gene density seen in *L. interrogans* confers properties for the successful survival, if not growth, in the outside environment. It may be recalled that *L. biflexa* lacks orthologs for LipL32, LipL41, HbpA, sphingomyelinases, Lig proteins, LipL21 and LipL36 seen in the pathogenic species. The vast wealth of data from genome analysis coupled with the modern tools for data analysis offers great opportunities for getting a better insight into the host-pathogen interactions.

### 3. Epidemiology

Leptospirosis is one of the most wide-spread zoonosis in the world. The global epidemiological data are extensively discussed elsewhere [Bharti et al., 2003; Levett, 2001]. The higher incidence in tropical countries is due to several reasons: one, the warm, wet and humid climatic conditions favour the survival and multiplication of the organisms; second, the close contact between the reservoir animal hosts and humans favour direct transmission between them and third, human practices such as walking barefoot and working in agricultural wet fields without protection allow indirect transmission from the contaminated environment. Rodents serve as major reservoirs of infection and rodent control must be considered as one of the important control measures. Pathogenic leptospires from the animal reservoirs are shed via the urine into the immediate environment. They can be transported from the immediate site of deposition to other areas during rainy / monsoon seasons; the heavy and torrential rains not only spread these organisms to new locations but also aid their survival in the environment for long periods of time. A reservoir of infection is thus established, with the movement of the infecting serovars among rodents, animals and humans; the latter are only accidental hosts and do not contribute to the transmission of the disease.



### 3.1 The Indian scenario

Historically, leptospirosis is not new to India with the disease reported in the 1930s in the Andaman & Nicobar islands [Taylor and Goyle, 1931]. In the 1960s, there were occasional reports of leptospiral etiology as seen in patients with PUO (Pyrexia of Unknown Origin) in whom the disease was attributed to the leptospiral serovars Icterohaemorrhagiae, Pomona and Canicola [Joseph and Kalra, 1966] and in a case of hepatitis due to *L. pyrogenes* [Bhatnagar et al., 1967]. In India, the incidence of the disease closely links to the rainy / monsoon seasons with higher incidence in the coastal states.

In South India, the states of Tamil Nadu and Kerala have reported several outbreaks of the disease, especially during rainy seasons [Natarajaseenivasan et al., 2004; Ratnam et al., 1993; Ratnam et al., 1983a; Ratnam et al., 1983b, 1986; Ratnam et al., 1987a; Ratnam et al., 1983c; Ratnam et al., 1987b; Sumathi et al., 2008]. The state of Kerala, called the Venice of the East, receiving heavy rainfall during several months of the year shows high prevalence of the disease, with the districts of Kottayam, Alleppey and Kozhikode being the worst affected [Vijayachari et al., 2008]. Patients present symptoms including history of fever, vomiting, jaundice, abdominal pain [Venkataraman et al., 1991], renal dysfunction [Muthusethupathi et al., 1995] and the involvement of the eye [Priya et al., 2007; Priya et al., 2008; Rathinam, 2002; Rathinam et al., 1997]. In 1994, there was an outbreak of leptospiral uveitis at Aravind Eye hospital, Madurai following severe flooding of the state of Tamil Nadu in the autumn of 1993; out of 46 patients, 80% of them were positive for leptospiral DNA and 72% were positive by serological tests. These studies clearly showed that leptospirosis is a significant health problem in coastal Tamil Nadu.

Other coastal regions include Mumbai, a heavily populated part of the country on the west coast of India. This city receives heavy rainfall during monsoon seasons and due to poor sanitation and unhygienic conditions is prone to the disease [Bal et al., 2002; Bharadwaj et al., 2002]. Several deaths due to leptospirosis were reported after the serious outbreak in 2005 [Sehgal, 2006]; around 310 cases of leptospirosis, with 27 deaths were reported, giving an incidence of 7.85 per 0.1 million population and a case fatality rate of 8.7%. The outbreak during the 2005 flooding in Mumbai clearly demonstrates the need for a proper surveillance and control measures during such times of need.

Leptospirosis is endemic in the Andaman Islands. After the report in 1931 [Taylor and Goyle, 1931], there were no reports of the disease till 1988. Following the monsoon of 1988, a mysterious febrile illness with hemorrhagic manifestations, named as 'Andaman haemorrhagic fever' (AHF) caused several deaths. Unfortunately for about 5 years the causative organism was not identified and it was in 1993 [Sehgal et al., 1995] the leptospiral etiology was identified. This important finding and several other notable contributions [Roy et al., 2003; Sehgal et al., 1999; Sehgal et al., 2000; Sharma et al., 2006; Sharma et al., 2003; Vijayachari et al., 2003] have been made by the National Leptospirosis Reference Centre located at Port Blair, Andamans. Pulmonary involvement in leptospirosis, observed in outbreaks in this part of India has been attributed to strain Valbuzzi serovar Valbuzzi of serogroup Grippotyphosa [Vijayachari et al., 2003]. Today, the disease is endemic and the whole population is at risk due to the climatic conditions and the occupation of the people living in these islands. There is high seroprevalence in manual laborers working in agricultural lands, sewage workers, animal handlers, personnel in slaughterhouses, making this disease a truly occupational nightmare.

In the state of Andhra Pradesh, there is no systematic study on human leptospirosis and the disease remains largely under-reported. We conducted a retrospective study in a hospital in Hyderabad, capital of Andhra Pradesh [Velineni et al., 2007]. 55 sera samples from patients who came from nearby villages with symptoms of leptospirosis were screened by MAT, Lepto-Tek Dri Dot and IgM ELISA. MAT identified 90% of the cases as positive and Icterohemorrhagiae emerged as the prevalent serogroup (68%). We also conducted a seroprevalence study in cattle in Mahbubnagar district, located about 100 km from Hyderabad. Agriculture is the major occupation in several villages in this district and the farmers live in close contact with the domestic animals that serve as a good source of income by way of milk and meat. We screened 107 sera from cattle from the villages of Ankur, Rajanagaram and Palakonda in this district and observed an overall seroprevalence of approximately 36.4% (Fig. 3a); the predominant serovar was Pomona (Fig. 3b).

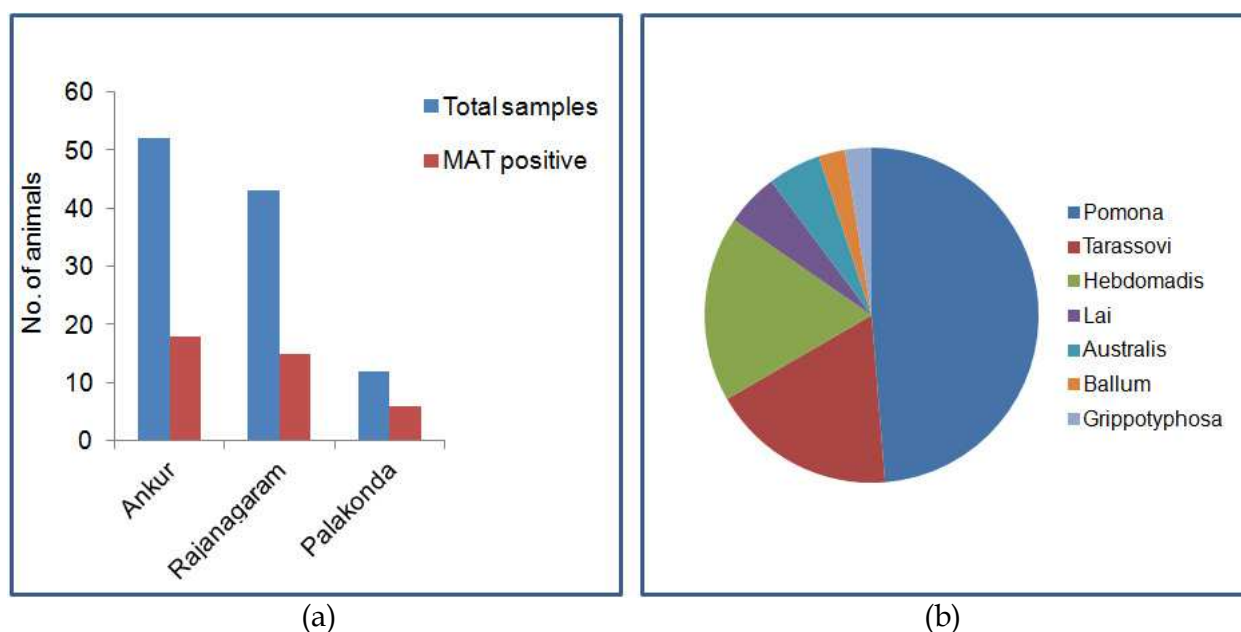


Fig. 3. Seroprevalence of leptospirosis in Mahbubnagar district. Panel (a) shows the MAT positive samples in the villages of Ankur, Rajanagaram and Palakonda in Mahbubnagar district in Andhra Pradesh. Panel (b) shows Pomona as the prevalent serovar (funding for the study by the Andhra Pradesh- Netherlands Program is acknowledged).

There are no reports of leptospirosis from the states of Jammu and Kashmir. Also, in general, there were few cases of the disease in North India due to the relatively dry weather. But now, a detailed study [Sethi et al., 2010] project the alarming increase in the incidence of the disease. The study aims to correlate predisposing factors like living conditions, human habits, exposure to animals, occupation etc to the observed disease prevalence as well as the severity of symptoms in these patients. An alarming increase in the severity of symptoms with the involvement of the liver and kidneys was seen. This may reflect a changing disease pattern in the Indian subcontinent or merely better diagnostic measures. There is a need for systematic and planned study to understand the prevalence of the disease, identification of the predominant serovars and recording of the symptoms associated with the disease. This will help to develop suitable control measures, especially in times of outbreaks.

#### 4. Clinical features

It is beyond the scope of this review to detail the clinical features of leptospirosis in animals. The clinical manifestations in humans are discussed briefly. In humans, it is not easy to diagnose clinically because of the large spectrum of symptoms associated with it that overlaps other diseases, especially in tropical countries. The disease presents itself first as the acute phase that may progress into the severe phase. In a vast majority of patients, it is usually self limiting and the infected individuals recover from the acute phase symptoms of fever, headache, chills and severe myalgia. As these symptoms overlap with viral flu (influenza), malaria and dengue, the patients are often treated symptomatically initially. The majority of patients recover without other complications, but in 5 -10% of the patients, the disease can progress into the severe Weil's disease. This is the hemorrhagic or icteric form of the disease [Levett, 2001] involving several organs including the liver, lungs and kidneys; the patients quickly deteriorate and the disease is often fatal.

The involvement of the liver is associated with jaundice with high levels of serum bilirubin. The jaundice is attributed to be due to the failure in the secretion of the bilirubin into the bile canaliculi and not due to hepatocellular necrosis [Bharti et al., 2003]. Acute renal failure, resulting in oliguria often leads to death. In patients with the classical AHF (Andaman Hemorrhagic Fever) symptoms, hemoptysis leads to adult respiratory distress syndrome. This disease also causes damage to the heart. In India, there are well documented evidences for the involvement of the eye in leptospirosis. Patients attending the Uvea Clinic in Aravind Eye Hospital have been diagnosed as suffering from leptospiral uveitis with symptoms including conjunctival suffusion and muscle tenderness that can result in blindness.

Treatment includes parenteral administration of benzyl penicillin (5 million units) per day for five days. Doxycycline can also be administered 100 mg twice daily for 10 days [Vijayachari et al., 2008]. It is however important to note that timely treatment is required for the prompt elimination of the pathogen before the disease progresses into the second phase leading to hemorrhage and tissue damage in the vital organs. This emphasizes clearly the need for timely diagnosis.

#### 5. Pathogenicity and virulence

In the majority of individuals, leptospirosis is often self-limiting as the host immune system efficiently clears the invading *Leptospira*. But, in a small percentage of infected individuals, the clinical manifestations can progress to the severe Weil's disease with jaundice, renal failure and potentially lethal pulmonary haemorrhage. While the pathogenesis and tissue damage is well documented in leptospirosis, mechanistic details like the pathogenic virulence determinants, their expression within the mammalian host and the damage inflicted due to immune mechanisms of the host are not completely understood. Extensive information on host tissue damage, involvement of specific leptospiral proteins and the host immune response are available [Bharti et al., 2003; Ko et al., 2009; Levett, 2001]

While 'pathogenicity' refers to the ability of a pathogen to cause disease, the term 'virulence', often used interchangeably with pathogenicity, refers to the degree of damage caused by the organism. Pathogens have adapted to the hostile environment of the mammalian host and elaborate several host-directed components for their survival. It is now becoming increasingly evident that the expression of many of the toxins / virulence determinants of pathogenic bacteria is environmentally regulated in response to specified

conditions. One of the contributing factors to virulence is iron limitation as the mammalian limits iron to an invading pathogen and the latter not only adapts to acquire this essential micronutrient but also expresses toxins upon iron limitation [Sritharan, 2000]. Iron is an essential micronutrient for *Leptospira* and they fail to grow in the absence of iron in culture media [Faine, 1959]. Nothing was known about how iron is acquired by these organisms, especially under conditions of iron deprivation. We first reported the high affinity transport system in *L. interrogans* serovar Lai [Asuthkar, 2007; Sritharan et al., 2005] and studied the role of iron as a regulatory molecule in the expression of the virulence factors sphingomyelinases [Velineni et al., 2009].

## 5.1 Adaptation of *Leptospira* to iron limitation

### 5.1.1 Low bioavailability of iron

Iron oscillates between ferrous ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) states and by virtue of its wide redox potential plays an important role in biological systems. It transfers reducing equivalents in the electron transport chain and acts as a cofactor for several enzymes in biochemical reactions. Most bacteria, including *Leptospira* require iron for growth. However, the inherent insolubility of the metal ion at biological pH makes it unavailable to bacteria, as it exists as insoluble ferric hydroxides and oxyhydroxides. Nature has perhaps made iron highly insoluble, as excess iron is toxic, due to its catalytic role in the Fenton reaction, resulting in the formation of free radicals [Sritharan, 2000]. At physiological pH, the major form of iron is  $\text{Fe}(\text{OH})_2^+$  with a solubility of approximately  $1.4 \times 10^{-9}$  M [Chipperfield, 2000] that is too low to support the growth of microorganisms. The mammalian host further limits iron to pathogenic bacteria by holding the metal ion as protein-bound iron; most of the free iron is bound by transferrin and lactoferrin and the excess iron is stored as ferritin.

### 5.1.2 Infection, iron-withholding & 'nutritional immunity' of the mammalian host

The mammalian host limits iron to an invading pathogen by a process called 'nutritional immunity' [Kochan, 1976]. In response to infection, the mammalian host lowers the level of circulating iron by decreasing the intestinal absorption of iron and increasing synthesis of transferrin. Other mechanisms [Weinberg, 2009] aimed at depriving iron to the actively multiplying pathogen include increased synthesis of hepcidin and lipocalin, the former inhibiting the release of iron by macrophages and the latter inhibiting bacterial growth by binding to the bacterial siderophores. Haptoglobulins and hemopexins are proteins expressed in the liver that come into play during conditions such as hemorrhage when there is high level of hemoglobin the circulation. While haemoglobin constitutes nearly two-thirds of the total iron in the human body, it is not readily available to pathogens because of its compartmentalization within the erythrocytes. When the level of free haemoglobin increases in the circulation, consequent to host cell lysis by bacterial toxins, the mammalian host immediately responds by triggering increased synthesis and release of haptoglobin and hemopexin in the liver as a part of the host defense mechanism [Sassa and Kappas, 1995].

### 5.1.3 How do *Leptospira* acquire iron?

Bacterial pathogens have evolved novel machinery to acquire the tightly-bound iron from the mammalian host. Box 3 summarizes the two general strategies for acquiring this essential micronutrient.



**Box 3: Bacterial adaptation to iron limitation**

High affinity bacterial iron acquisition systems include

- a) **Siderophore-mediated system:** Siderophores are Fe<sup>3+</sup>-specific low molecular weight ligands (~1000 Da) that chelate iron from the immediate environment (from sources including insoluble iron and protein-bound iron in the mammalian host) and deliver the metal ion to the organism by active transport mediated by specific cell-surface receptors called iron-regulated membrane proteins.
- b) **Direct acquisition:** Pathogenic bacteria directly chelate Fe<sup>3+</sup> via specific cell-surface receptors for host transferrin / lactoferrin / heme / hemoglobin.

(for detailed reviews see [Braun et al., 1998.; Sritharan, 2000])

*Leptospira* do not produce siderophores, suggesting that direct acquisition from the host iron-containing molecules must occur. We identified the hemin-binding protein HbpA and demonstrated iron acquisition from hemin. First, using *in silico* tools we identified HbpA as the leptospiral homologue of the ferric enterobactin receptor *FepA* of *E. coli*. Structural elucidation and bioinformatic analysis confirmed the protein to be a TonB-dependant protein with the TonB box in its N terminal region [Sritharan et al., 2005]. As in other bacterial iron transporters, HbpA possibly transports the hemin molecule via the TonB system, comprising of the cytoplasmically localised TonB, ExbB and ExbD proteins; the TonB protein, extending through the periplasm to the outer membrane mediates the transfer of the proton motive force of the cytoplasmic membrane to the outer membrane iron transporters, followed by the internalization of the Fe<sup>3+</sup>- complexes. 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*, than can degrade the tetrapyrrole ring of the heme molecule, thereby releasing ferrous iron.

The hemin-binding property of HbpA was proved in several ways [Asuthkar, 2007]. The presence of the conserved FRA/PP-NPNL motif in the primary sequence of the protein reflected its ability to bind hemin. Experimentally, hemin-binding was demonstrated both by assaying the inherent heme-dependent peroxidase activity of the bound hemin and by spectrofluorimetry. Addition of hemin resulted in the quenching of the emitted light and notably a spectral shift to light of a lower wavelength (blue shift), a characteristic feature of receptor-ligand binding.

HbpA is up-regulated by iron limitation and increase in temperature; both these conditions are noteworthy as they will be encountered by the pathogen inside the mammalian host. The expression of HbpA *in vivo* [Sridhar et al., 2008] and its absence in the non-pathogenic *L. biflexa* highlights its role in pathogenic *Leptospira*. It is not clear if all leptospiral species elaborate this mechanism of iron acquisition as the *hbpA* gene could not be detected by PCR in several leptospiral species. The latter probably express receptors for other host iron-containing proteins; we identified a transferrin-binding protein in iron-limited *L. kirschneri* serovar Grippotyphosa strain Moskva V (unpublished observations in our lab).

Intracellular iron acts at the molecular level to regulate the expression of the components of the iron acquisition machinery in bacteria [Sritharan, 2000, 2006]. In most Gram negative bacteria, iron binds to the Fur regulator (DtxR in Gram positive organisms) and this dimeric Fur-Fe<sup>2+</sup> complex binds to Fur / iron box with the consensus sequence 5'-GATAATGATAATCATTATC present upstream of iron-regulatable genes. Iron must act via the Fur protein in *Leptospira* as they show the presence of Fur homologs and Fur box.

Several *fur* genes are present in the serovar Lai that however remains to be experimentally proved as iron regulators. Table 1 lists the *fur* genes in serovar Lai and their corresponding homologues in the other leptospiral species. Fur, encoded by LB183, one of the *fur* genes present in the vicinity of *hbpA* (LB191) possibly acts by binding to the Fur box (5' GATAATCATAATAATTT) located upstream of *hbpA* [Sritharan et al., 2005].

<i>Leptospira interrogans</i>		<i>L. borgpetersenii</i>		<i>L. biflexa</i>
		Serovar Hardjo-bovis <sup>3</sup>		
Serovar Lai <sup>1</sup>	Serovar Copenhageni <sup>2</sup>	Strain L550	Strain JB197	Serovar Patoc <sup>4</sup>
LB183 (II)	LIC 20147 (II)	-	-	LEPBI_I2152 (I)
LA 3094 (I)	LIC 11006 (I)	LBL_2245 (I)	LBJ_0837 (I)	LEPBI_I2330 (I)
LA1857 (I)	LIC 12034 (I)	LBL_1818 (I)	LBJ_1600 (I)	LEPBI_I2461 (I)
LA2887 (I)	LIC11158 (I)	LBL_1012 (I)	LBJ_2038 (I)	-
-	-	-	-	LEPBI_I2849 (I)

Table 1. The *fur* genes in *Leptospira* spp.

- References represented by 1, 2, 3 & 4 include [Bulach et al., 2006; Nascimento et al., 2004; Picardeau et al., 2008; Ren et al., 2003].
- The *fur* genes, as annotated in the respective genomes are represented by their locus tag and the chromosome in which they are present is indicated within parenthesis. When the corresponding orthologue is absent, it is indicated by (-) sign.

## 5.2 Iron levels and expression of the leptospiral sphingomyelinases

The ability of pathogens to utilize heme compounds is particularly important as heme is one of the most abundant forms of organic iron in animals. Acquisition of iron from heme or hemoglobin may be facilitated by the production of hemolysins or cytotoxins which lyse host cells and release the intracellular iron. Cytotoxin production coupled with the capability to utilize heme and/or hemoglobin could serve as an effective iron acquisition strategy during the progression of infection. It is well known that leptospires cause localized damage to the endothelium of the small blood vessels that leads to severe damage in tissues like kidneys, liver and lungs. The necrosis in the renal tubules, hepatocellular and pulmonary hemorrhage is irreversible and is often fatal.

### 5.2.1 Leptospiral hemolysins

The hemorrhage and the ensuing symptoms observed in leptospirosis are due to the lysis of the host cells by the hemolysins. The hemolytic activity of these toxins was reported as early as 1956 [Alexander et al., 1956]. Later, several researchers implicated these molecules in the pathogenesis of the disease [Bernheimer and Bey, 1986; del Real et al., 1989; Kasarov, 1970; Segers et al., 1990; Thompson and Manktelow, 1989]. The phospholipase and sphingomyelinase activities of these molecules were clearly demonstrated in some of the studies. However, it was not easy to correlate all these findings as they were variously called and demonstrated in different serovars. Today, with the information from genome data

there is clarity on the types and numbers of hemolysin genes in the two pathogenic species and the saprophytic *L. biflexa*.

There are nine hemolysin genes in *L. interrogans* serovar Lai (Table 2) encoding four sphingomyelinase, one pore-forming and four non-sphingomyelinase types of hemolysins. Fig. 4 shows the corresponding orthologues in *L. borgpetersenii* serovar Hardjo and it may be noted that it lacks the pore-forming hemolysin SphH. The sphingomyelinase orthologs are absent in the non-pathogenic *L. biflexa*, suggesting that their expression in the pathogenic *Leptospira* are likely to confer additional advantages for their survival within the mammalian host. The role of the five non-sphingomyelinase genes in *L. biflexa* (LEPBla0082, LEPBla0717, LEPBla2015, LEPBla2375 & LEPBla2477) is however not clear.

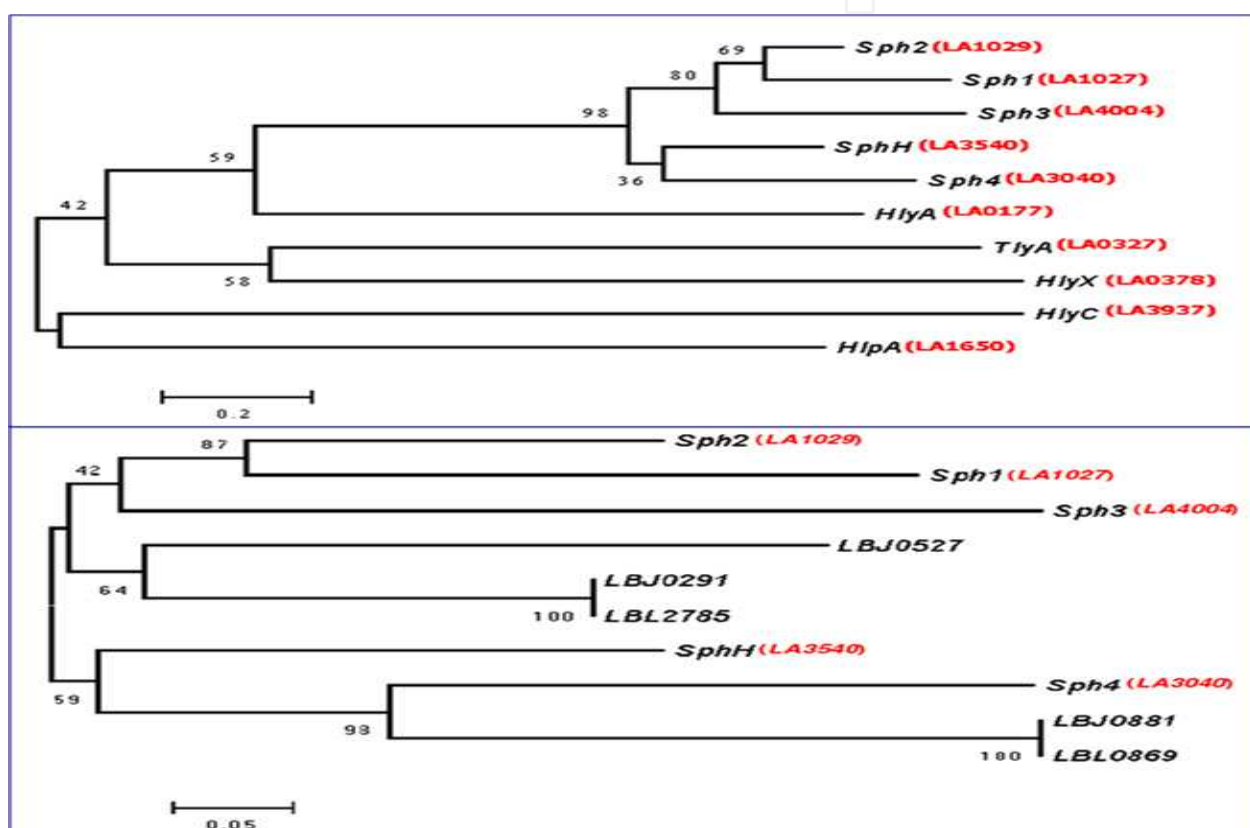


Fig. 4. Phylogenetic analysis of leptospiral hemolysins. Panel (a) shows the phylogenetic tree of the sphingomyelinase and the non-sphingomyelinase hemolysins of *L. interrogans* serovar Lai and Panel (b) shows the corresponding orthologues in *L. borgpetersenii* serovar Hardjo (strains L550 and JB197). The tree was generated using Clustal X and Mega 3.1 software. The phylogenies generated by neighborhood joining with 400 bootstrap replicates, rooted at midpoint and bootstrap values, are shown as percentages. The numbers refer to the divergence between the sequences.

There are reports on the biological activity of recombinant hemolysins including the hemolytic, enzymatic (sphingomyelinase and phospholipase) and pore-forming activity of these molecules [Artiushin et al., 2004; Lee et al., 2000; Lee et al., 2002]. Studies in our lab are also focused in the structural elucidation and functional characterization of the sphingomyelinases (under communication).

Type of hemolysin	Gene	Locus Tag	Approximate molecular mass (kDa)
Sphingomyelinases	<i>sph1</i>	LA1027	68.19
	<i>sph2</i>	LA1029	71.03
	<i>sph3</i>	LA4004	65.33
	<i>sph4</i>	LA3050	27.92
Pore-forming hemolysin	<i>sphH</i>	LA3540	64.43
Non-sphingomyelinase hemolysins	<i>hlyC</i>	LA3937	50.53
	<i>hlyX</i>	LA0378	44.95
	<i>tlyA</i>	LA0327	31.67
	<i>hlpA</i>	LA1650	36.53

Table 2. Hemolysins in the genome of *L. interrogans* serovar Lai [Ren et al., 2003]

### 5.2.2 Expression of sphingomyelinase(s) in *L. interrogans* serovar Lai upon iron limitation

In *L. interrogans* serovar Lai, a 42 kDa protein was detected in the outer membrane vesicles (OMVs) of low iron organisms that was absent in the OMVs from high iron cultures [Velineni et al., 2009]. This protein was recognized by antibodies against the common domain shared by the sphingomyelinases Sph1, Sph2 and Sph3 (Fig. 5).

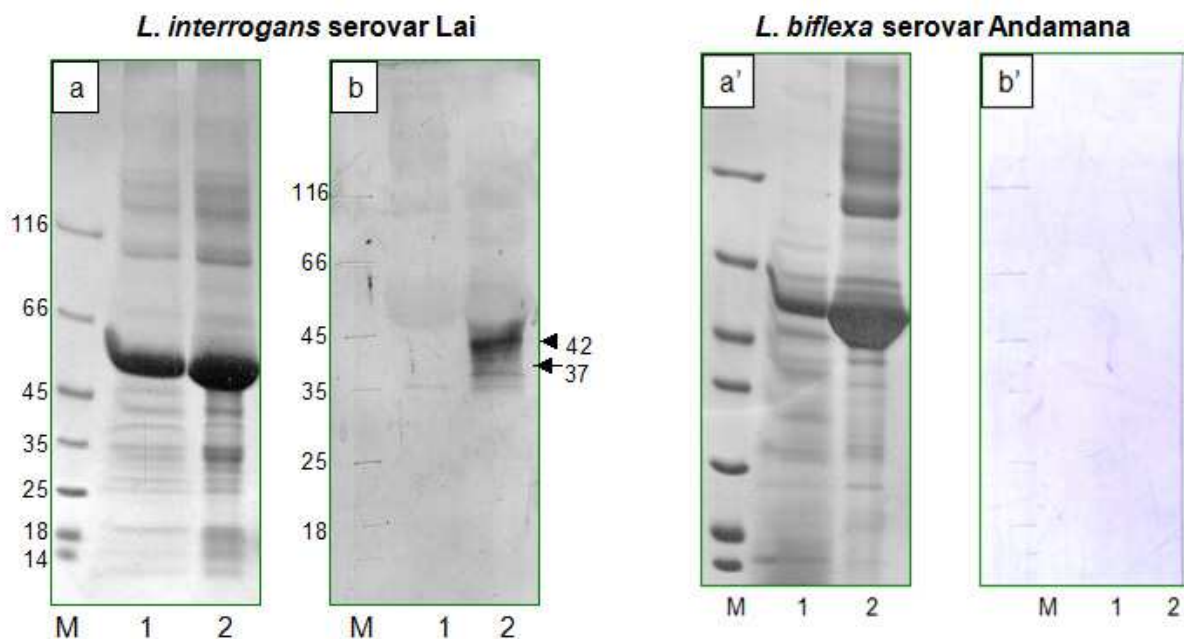


Fig. 5. Expression of sphingomyelinase in iron-limited serovar Lai. Anti-sphingomyelinase antibodies identified a major 42 kDa band in the OMVs of low iron serovar Lai (lane 2, Panel b); the band was absent in OMVs of high iron organisms (lane 1, Panel b). The Panel (a) shows the corresponding SDS-PAGE profile of high and low iron organisms. The panels a' and b' represent the corresponding samples from the non-pathogenic *L. biflexa* that does not show any reactivity with anti-sphingomyelinase antibodies. (Reproduced with permission from Online Journal of Bioinformatics)



As all the three sphingomyelinases encode bigger products, it clearly indicates that the 42 kDa protein is a cleavage product. This raises several questions, including the role of iron in the expression of one or more of these sphingomyelinase precursors, the proteolytic cleavage of the precursor and functional characterization of the 42 kDa protein.

Anti-sphingomyelinase antibodies, primarily used in the pull-down assay for the identification of sphingomyelinases from outer membranes, surprisingly led to the identification of the 63 kDa efflux protein TolC encoded by LA0957 [Velineni et al., 2009]. Structurally it is identical to the  $\alpha$ -hemolysin-transporting TolC protein of *E. coli* [Balakrishnan et al., 2001]. Based on the structural similarity and the presence of the associated ATP-binding protein HlyB (LA0150) and HlyD (LA3737) in the leptospiral genome, we predict that the leptospiral TolC acts as a transporter of sphingomyelinase. However, unlike the cistronic organization of the genes *hlyCABD* responsible for  $\alpha$  hemolysin (HlyA) secretion seen in *E. coli*, the leptospiral *hlyB* and *hlyD* are not organized as an operon. This is similar to the organization seen in *Neisseria meningitidis* in which *hlyD* and *tolC* genes are adjacent but unlinked to *hlyB*, with the three genes being expressed independently (Wooldridge et al., 2005).

## 6. Diagnosis

### 6.1 Leptospiremic or antigenic phase: Culture, DFM and PCR

The organisms gain entry into a host through skin via cuts or abrasions or mucous membranes such as the conjunctiva. They enter the blood circulation where they can be detected for about a week. This period, called the septicemic or leptospiremic phase allows the direct detection of the pathogen either by culture, dark field microscopy or by molecular methods such as PCR (Fig. 6). Culture can be done by directly inoculating semi-solid media

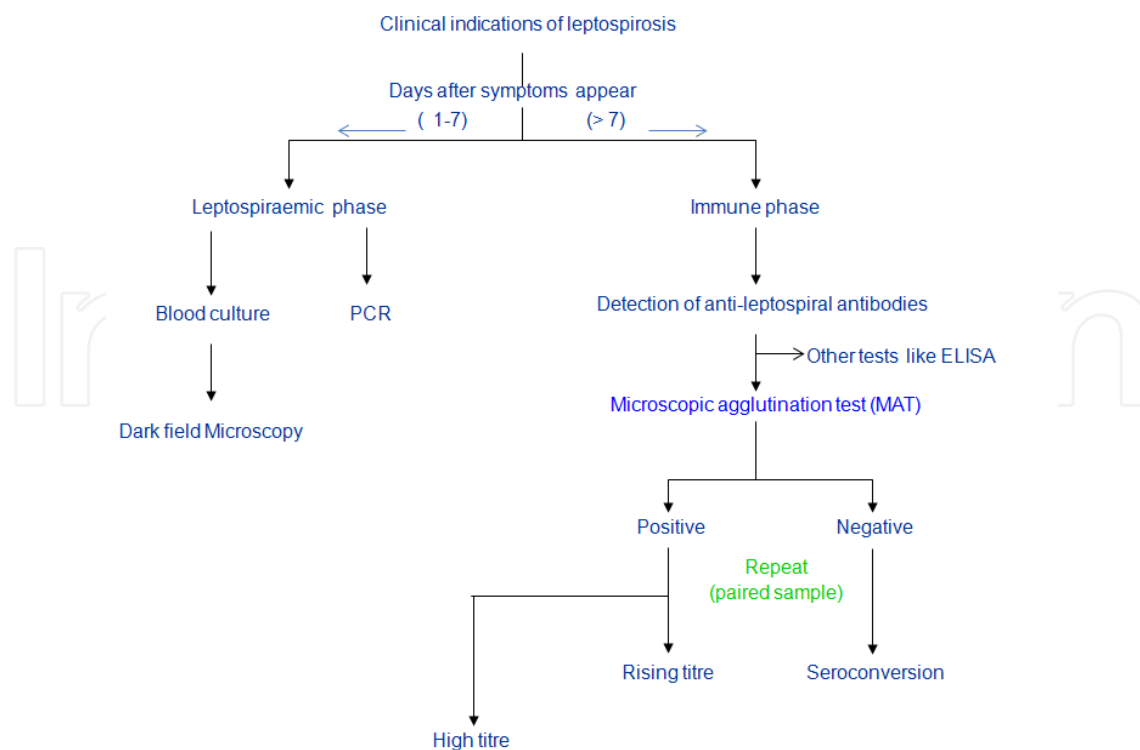


Fig. 6. Diagnosis of leptospirosis – a schematic representation.

with a few drops of blood, care taken not to include citrate as an anticoagulant as it inhibits growth of the organisms (Wolff, 1954). The media should be inoculated within 24 hours upon collection and incubated at 28 - 30°C for several weeks. The main disadvantage of blood culture is that it requires several weeks of incubation, has low sensitivity and is not useful during epidemics. Dark field microscopy, though reported as a method for diagnosis is not advisable as artifacts in biological samples can be mistaken for the organisms, leading to false positivity.

Molecular diagnosis by PCR has been used as a tool to detect pathogenic leptospire in biological fluids. Several targets specific for pathogenic serovars have been used. One of them is the 16s rDNA from which a 631 bp product [Hookey, 1992] or a 330 bp product (Senthilkumar *et al.* 2001) can be amplified. A real-time PCR method based on 16S rDNA was developed that could be used on samples without the need for prior isolation and culture [Smythe *et al.*, 2002]. Another popularly used method [Gravekamp *et al.*, 1993] is based on 2 sets of PCR primers, namely G1 / G2 and B64-I / B64-II that amplify products of 285 bp and 563 bp respectively. The former detected *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri*, while the later identified *L. kirschneri*. Other variations included 'Magnetic Immuno PCR Assay' (MIPA) [Taylor *et al.*, 1997] that consists of the immuno-magnetic separation of leptospire from inhibitors in frozen formalin-fixed bovine urine prior to PCR detection that resulted in a marked improvement in the detection of leptospire in urine samples. PCR based on *ompL1* [Reitstetter, 2006] detected serovars belonging to *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. santarosai*, *L. weilii* and *L. noguchii*. We used *hbpA* as a target for PCR [Sridhar *et al.*, 2008] and detected all serovars belonging to *L. interrogans* from clinical isolates obtained from different geographical locations around the world. A detailed study to identify the presence of *hbpA* in other species, sequence analysis and the design of suitable primers is required to use *hbpA* as a target for the identification of other leptospiral species.

## 6.2 Immune phase: Assay of anti-leptospiral antibodies by MAT, ELISA, lateral flow devices

High levels of anti-leptospiral antibodies of the IgM class appear 7-10 days after infection (Fig. 6) as a strong humoral immune response is mounted by the mammalian host [Fennestad and Borg-Petersen, 1957; Fennestad *et al.*, 1968; Levett, 2001; Ratnam *et al.*, 1983b; Ratnam *et al.*, 1983c]. The mammalian host strives to eliminate the pathogen via the antibody-complement system. However, the leptospire may migrate into the organs, with a predilection for liver, lungs and kidneys. In the latter, they may settle in the convoluted tubules of the kidneys thus evading the antibody-complement system of the host. In humans and more characteristically in animals, where the infection tends to be chronic, leptospire are shed via urine. Thus detection of the pathogen in the urine by dark field microscopy, PCR or culture may be attempted, though it may be noted that the shedding is not uniform and occurs randomly and intermittently, with periods of nil shedding, when the testing for the pathogen will yield negative results.

The antibody response is classical, with peak IgM levels appearing first, followed by IgG antibodies. The IgM antibodies however remain in circulation for considerably long periods, even upto 2 months. It has also been observed that anti-leptospiral antibodies can be detected even after several years of infection. This, coupled to the endemicity of the

infection may result in relatively high levels of antibodies within a population. Thus, it is common to collect a second serum sample from a suspected case of leptospirosis, 3 - 4 days after the first sample. Sero-conversion with a four-fold rise in titre in paired serum samples in the presence of clinical symptoms is an important criterion for the definitive diagnosis of leptospirosis. Anti-leptospiral antibodies can be detected by several methods, including the microscopic agglutination test, ELISA, lateral flow devices, latex bead agglutination tests etc.

### 6.2.1 Microscopic agglutination test

Microscopic agglutination test (MAT) is the 'gold standard' for leptospiral diagnosis [Cumberland et al., 1999]. This highly specific and sensitive test is based on the agglutination of live organisms in the presence of serum containing anti-leptospiral antibodies. The agglutination results in the formation of highly refractive spheroids of various sizes and with time, when maximal degree of agglutination is seen, no free leptospores are visible due to the disintegration of the organisms. The degree of agglutination is usually assessed in terms of the proportion of free leptospores. The accepted endpoint of an agglutination reaction is the final dilution of serum at which 50% or more of the leptospores are agglutinated. As per WHO guidelines, agglutination at dilution of 100 is considered positive for MAT. Antibodies in the serum of infected patients / animals, predominantly against the surface-exposed lipopolysaccharides are serovar-specific, although cross-reactivity may be recorded against other serovars within the same serogroup. It is thus necessary to include several serovars, including the prevalent local isolates for screening by this method. As mentioned earlier, paired samples are to be considered for diagnosis. However, a positive diagnosis can also be made with a titre of more than 800 with single samples [Ko et al., 2009].

MAT has been used as the test of choice in outbreaks and sporadic cases. It has also been useful in retrospective studies in confirming leptospirosis cases and identifying the prevalent serovar during that period. Ismail *et al.* (2006) used MAT in a retrospective study on serum samples from patients with undiagnosed acute febrile illness (AFI) and hepatitis cases from Egypt and showed leptospiral etiology in 16% of AFI (141/886) and 16% of acute hepatitis cases (63/392). A retrospective hospital-based study done in our lab [Velineni et al., 2007] on serum samples collected from suspected cases of leptospirosis identified Icterohaemorrhagiae as the predominant serogroup.

MAT can be used to study the seroprevalence in domestic animals. In a study on 424 sow's sera from the Mekong delta in Vietnam [Boqvist et al., 2002], including 283 sows from small-scale family farms and 141 from large-scale state farms, the overall seroprevalence was 73 and 29% respectively; the predominant infecting serovars in the respective farms were *L. interrogans* serovar Bratislava and *L. interrogans* serovars Icterohaemorrhagiae. As mentioned earlier, in a seroprevalence study on cattle in Mahbubnagar district in the state of Andhra Pradesh, MAT identified Pomona as the predominant serovar (Fig. 3).

### 6.2.2 Enzyme Linked Immunosorbent Assay (ELISA)

Antigens used in ELISA include whole cell sonicate, formalin-extract of a culture of leptospores [Terpstra et al., 1985] and whole leptospores coated on polystyrene microtitre plates [McBride et al., 2007]. Outer membrane proteins like rLipL32 [Fernandes et al., 2007; Flannery et al., 2001] , rLipL41 [Flannery et al., 2001; Mariya et al., 2006] and

immunoglobulin (Ig)-like Lig proteins (Croda *et al.*, 2007; Srimanote *et al.*, 2008) have been used as antigens in ELISA.

Antibodies against the iron-regulated hemin-binding protein HbpA are present in the serum of patients with leptospirosis [Sridhar *et al.*, 2008]. It shows considerable potential as a candidate antigen in ELISA for the screening of sera from cattle and in humans with leptospiral uveitis (both under communication). 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 and further, unlike MAT, does not require trained personnel and can be quantitated and is not prone to inter-observer and intra-observer errors as in MAT.

## 7. Future perspectives

Timely diagnosis of leptospirosis will result in a significant reduction in the mortality. This is possible with better insights into the pathogenesis and host-pathogen interactions. The great strides made in the past decade in understanding the basic biology has certainly broadened our efforts and approaches towards better control measures. Studies in our lab are focused on the development of a simple, easy-to-do ELISA test based on anti-HbpA antibody detection for sero-diagnosis. This will facilitate screening for the disease, not only in well-established hospitals and labs but also in rural centers for the early diagnosis of the disease.

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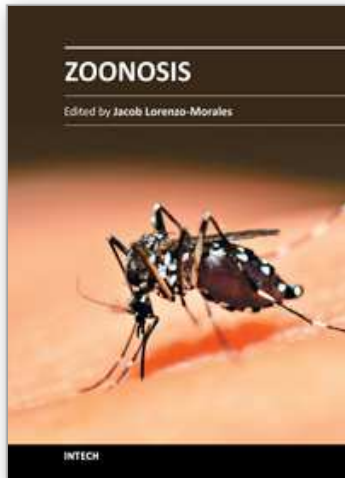


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Zoonotic diseases are mainly caused by bacterial, viral or parasitic agents although "unconventional agents" such as prions could also be involved in causing zoonotic diseases. Many of the zoonotic diseases are a public health concern but also affect the production of food of animal origin thus they could cause problems in international trade of animal-origin goods. A major factor contributing to the emergence of new zoonotic pathogens in human populations is increased contact between humans and animals. This book provides an insight on zoonosis and both authors and the editor hope that the work compiled in it would help to raise awareness and interest in this field. It should also help researchers, clinicians and other readers in their research and clinical usage.

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